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The foundation of the research compilation presented here began with the derivation of an improved method for obtaining optimal in vitro mitogenic responses in salmonid lymphocyte cultures by utilizing autologous or homologous plasma as the primary serum supplement. It was observed that lymphocytes which were previously unresponsive to mitogenic challenge *in vitro*, when cultured in fetal bovine serum, responded well when cultured in the presence of homologous plasma. Salmonid plasma sources not only repeatedly enhanced the mitogen-specific proliferation of the lymphocyte cultures, but enhanced the antibody response as well. A prolonged kinetic response further supported the contention that former conditions of salmonid lymphocyte culture, employing only fetal bovine serum, not only fail to provide the optimal conditions for cell growth, but in many cases the essential conditions. Enhancement of the mitogenic response was observed for three distinct species, using a common plasma source, suggesting utilization of plasma as an alternate serum supplement has broad applications and may be adapted to many fish systems.

With this improved culture system, examination of the regulation of lymphocytes, specifically B cells, was undertaken. Evidence for the existence of a natural regulatory cell population located in the anterior kidney is presented. Addition of anterior kidney cells to either autologous peripheral blood or spleen cell cultures resulted in significant suppression of the mitogen response. The degree of suppressor activity appears to be correlated with the anterior kidney lymphocyte's ability to respond to mitogenic stimulus. It is demonstrated that a decrease in the anterior kidney mitogen response correlates significantly with an increase in the suppressor activity observed upon coculture of these same cells with peripheral blood lymphocytes. Interestingly, while addition of anterior kidney cells to spleen plaque forming cell cultures also resulted in suppression, anterior kidney cells had either no effect or enhanced the antibody response of the peripheral blood lymphocyte cultures. It is postulated that the mediator of this anterior kidney activity is a suppressor cell population which may possess an important immunoregulatory function.

In Vitro Regulation and Cultivation of Salmonid Lymphocytes

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In Vitro Regulation and Cultivation of Salmonid Lymphocytes

CHAPTER 1

Introduction and Literature Review

INTRODUCTION

Since the initiation of studies using *in vitro* methods to examine immune responses, the importance of the cell's environment has been emphasized and thoroughly investigated. In order to analyze lymphocyte function as it occurs *in vivo*, the synthesized culture conditions used *in vitro* must approach the identical conditions the cell would be subjected to *in vivo*. The difficulty of this task is enormous and, to date, has not been perfected either in endothermic or ectothermic vertebrate systems. Probably the major stumbling block has been the shortcomings in defining the essential components of serum, a vital supplement for most cell culture work. Fetal bovine serum (FBS) has been used routinely as the preferred serum supplement due to initial studies comparing different serum sources for their ability to support cell growth.

In addition to the problems in defining essential components of serum, there are other factors present in serum which are toxic to cells, some of which have been identified, but many of which have not. Therefore, in several mammalian, as well as fish, systems fetal bovine serum has been

demonstrated to be inhibitory or often toxic to particular cell types. It may also be argued that the inhibitory effects of serum may not be due to the presence of toxic factors, but rather due to an absence of essential factors. Whatever the case, it is clear there is much room for improvement still today in optimizing cell culture conditions.

In the fish system, in vitro culture methods have been adapted from mammalian cell culture procedures employing fetal bovine serum as the primary serum supplement. The study of the fish immune system is interesting from both a comparative and evolutionary aspect. In this respect it seems quite contrary to utilize a xenogeneic, endothermic serum source to examine the immune system of an ectothermic species. The objectives of the research presented here were to optimize culture conditions for salmonid leukocytes by using homologous salmonid plasma as the primary serum supplement, and with an improved culture system, begin to reexamine lymphocyte function and the regulation thereof.

REVIEW OF LITERATURE

LYMPHOCYTE CULTIVATION

Origins of in vitro lymphocyte culture Cellular immunologists employ *in vitro* methods to investigate lymphocyte subpopulations and their roles in the generation and regulation of both specific and nonspecific immunity. The advantages of *in vitro* culture are many, including efficient measurement of multiple samples, ability to control desired variables, and use of a uniform suspension of cells for both experimental and control assessments (Mishell, 1980). Mishell and Dutton have been credited with the first successful attempt at stimulation and culture of a dissociated spleen cell suspension (Mishell and Dutton, 1966; Mishell and Dutton, 1967). Previous to 1965, the main lymphocyte culture methods employed to examine antibody production included test tube cultures (Pappenheimer, 1917; Schrek, 1943), slide chambers (Mackaness, 1952; Gowans, 1957), plasma clot cultures (Lewis and Webster, 1921; Maximow, 1928), and whole organ cultures (Trowell, 1952). Employing the latter culture methods, many claimed to observe secretion of antibody into the tissue culture medium, although inadequacies in these techniques permitted little or no proliferation of lymphocytes *in vitro* with or without antigen stimulation (Trowell, 1965).

Mishell and Dutton in 1966, demonstrated that spleen cells from normal, unimmunized mice, when cultured and immunized *in vitro*, could provide a response comparable to that seen *in vivo*. Soon after, Mishell and Dutton (1967) presented a more detailed account of the conditions for culture and immunization of the spleen cells. What they perceived as critical conditions included low oxygen tension, gentle agitation of the cultures,

inclusion of fetal bovine serum in the medium, adequate cell density, and daily feeding of the cultures with a nutritional mixture. Later, Click et al., (1972) further optimized the culture system by changing the medium components to eliminate the need for rocking the cultures and daily feeding.

Serum supplementation. The recognition of the importance of controlling and defining the *in vitro* cellular environment via manipulation of the medium is attributed to Lewis and Lewis (1911). Since that time, great energy has been devoted to the optimization of culture conditions for mammalian and other vertebrate cells. Waymouth (1972) outlined some of the historical landmarks in the development of tissue culture medium as we know it today. Burrows in collaboration with Carrel (Burrows, 1910; Carrel, 1912) introduced the use of clotted blood plasma as a support for explanted avian and mammalian tissues between 1910 - 1917. Their work was the basis for the observation that although a defined medium was adequate for some cell types, supplementation with serum was essential for others. Analysis of the components of serum responsible for this growth-promoting activity still continues today. The critical importance of the undefined factors present in serum led many to attempt isolation and characterization of these factors (Temin et al., 1972; Brooks, 1975). Unfortunately several of these attempts were unsuccessful.

The role of many macromolecular factors present in serum has, as yet, to be clearly defined. Many of the important properties of serum have been dissected and reviewed (Temin et al., 1972; Barnes, 1987). One role of serum or macromolecules may be as carriers of essential low molecular weight components. Such components likely include vitamins, amino acids, lipids and choline. Evidence for this role was demonstrated by showing that

these low molecular weight compounds could replace much of the requirement for serum in certain cell cultures. Other critical components of serum are hormones, many of which also are found bound to carrier proteins in serum (Barnes and Sato, 1980). Upon further review, Temin et al., (1972) concluded that although the supply of low molecular weight factors by serum may be important for some cells, macromolecular components must have an additional role in cell multiplication *in vitro*. Control of cell multiplication is highly dependent upon the availability of specific growth-promoting factors, and the levels of toxic or inhibitory factors. These factors can either be found in the serum or produced by the cells themselves.

Although fetal bovine serum (FBS) supplementation has provided immunologists with the means to study induction of *in vitro* immune responses, more recent analyses have revealed inconsistencies in FBS supplementation. Analyses of the role of serum specifically in the *in vitro* induction of immune responses demonstrated the effects of deficient fetal bovine serum (FBS) and the prevalence of these deficient batches of FBS (Watson and Epstein, 1973; Shiigi and Mishell, 1975; Shiigi et al., 1975). Shiigi and Mishell (1975) tested more than 200 samples of pooled FBS to determine their capacity to support primary immune responses by murine spleen cells. They discovered less than 10% of these samples were fully supportive; the rest were moderately to extremely deficient. They presented evidence that the supportive sera were the result of bacterial contamination. Sera known to have been temporarily contaminated during processing were highly supportive. As a result of this study, newborn calf serum (NBC) as opposed to fetal bovine serum, was examined as a potential alternative serum source for *in vitro* lymphocyte culture (Shiigi et al., 1975). Their data demonstrated NBC serum inhibited the *in vitro* generation of primary

antibody immune responses, but supported the *in vitro* generation of secondary humoral responses. It was also determined that sera obtained from newborn calves before nursing were not inhibitory for primary responses, but sera obtained from the same calves 7 days later did inhibit these responses. Thus they postulated that the inhibitory effects of NBC sera may be due to a physiological control mechanism which becomes active upon immunological maturity and may inactivate beneficial cell mediators.

As advances in understanding cellular requirements for growth evolved, development of chemically defined serum-free media for use in cell culture resulted. Use of semidefined media devised by eliminating step by step fewer identified serum components eventually led to fully chemically defined media, or serum-free media, among the earliest being those of White (1946). The advantages of using serum-free media include a clearer determination of how the interaction of hormones, drugs, and other factors may influence cell growth and differentiation, the definition of cellular nutritional requirements, and the specific examination of secretion of factors or cytokines by various cell types (Barnes and Sato, 1980; Darfler and Insel, 1984). According to Darfler and Insel (1984) lymphocytes have several unique attributes which have made attempts to develop serum-free media easier in some respects and more difficult in others. For example; lymphocytes require no factors for cell attachment, and no enzymatic treatment to facilitate passage of adherent cells. Difficulties arise, though, when one examines lymphocytic requirements for growth and/or differentiation. Lymphoid cells are known to require relatively large amounts of protein, such as bovine serum albumin, or casein. Additionally, these cells have a strict requirement for added lipids, at levels close to toxic for

these cells (Darfler and Insel, 1982). More recently, others have developed what they deem adequate serum-free culture methods for mammalian lymphocytes (Blaehr and Ladefoged, 1988; Ohmori, 1988; Rhodes et al., 1990; Strauss, 1988).

Serum in fish cell culture. Despite the apparent contraindications for the routine use of fetal bovine serum as a primary serum supplement, it is still one of the most commonly used serum sources. This has been the case not only for mammalian lymphocyte culture, but for most vertebrate systems. After initial experimentation and attempts at optimizing freshwater fish cell culture, Wolf (1973) composed a methods guideline for the development of media and optimal conditions for the culture of fish cells. Wolf states, "Without reservation, fetal bovine serum is the nutrient supplement of choice and a 10% level has generally been found to be a compromise between effectiveness and economy." In fact the methods and growth media used in fish cell culture have been adapted and generally do not differ from those used for the culture of cells from homeothermic vertebrates (Wolf and Mann, 1980; Wolf and Ahne, 1982; Nicholson, 1989). Thus, fetal bovine serum has been routinely used as the preferred serum supplement.

Alternatives to fetal bovine serum have been investigated for use in leukocyte culture in some of the warm water fish species (Cuchens and Clem, 1977; Clem et al., 1981; Rosenberg-Wiser and Avtalion, 1982; Faulmann et al., 1983; Caspi et al., 1984). Determination of optimal serum supplements for catfish and bluegill lymphocyte culture included the testing of human, calf, rabbit, alligator, bass, grouper, bream, and catfish sera or plasma (Cuchens and Clem, 1977). Bluegill lymphocytes responded best to bream plasma whereas Faulmann et al. (1983) observed little functional

activity unless a combination of 10% human plasma and 5% channel catfish sera was employed. Additionally, Avtalion and his coworkers examined various sera and determined that 2-4% charcoal absorbed carp serum was optimal (Rosenberg-Wiser and Avtalion, 1982; Caspi et al., 1984).

Studies with the salmonid species, however, have not uniformly demonstrated an optimum serum supplement for leukocyte cultures. Etlinger et al. (1976) demonstrated that 20% rainbow trout (RT) serum enhanced mitogenesis when compared to FBS, but this was not adopted by other laboratories. Rather, studies of salmonid species have continued to utilize FBS as the primary serum supplement (Warr and Simon, 1983; Blaxhall, 1985; Tillit et al., 1988). Preferential utilization of FBS may partially be due to observations of inhibitory effects of trout serum on leukocyte cultures (Blaxhall, 1985). However, additional studies on trout mitogenesis have shown that FBS can yield varying results, from spontaneous mitogenic activity to an inability to support mitogen-induced reactivity (Etlinger et al., 1976a; Tillit et al., 1988; DeKoning and Kaattari, 1991).

More recently, as in mammalian studies, serum-free media have been investigated for use in fish cell culture. Shea and Berry (1983) were the first to adapt a serum-free media, previously developed for mammalian cells, for use in the culture of non-lymphoid fish cell lines. The most recent report, and possibly the only report, of the use of a serum-free media in fish lymphocyte culture is presented by Luft et al. (1991). In this study, two commercially available serum-free media, when combined, were reported to contain the requisite nutrients for short term proliferation and differentiation of channel catfish T and B lymphocytes.

LYMPHOCYTE REGULATION

Mammalian B cell ontogeny. It has been well documented that the sites of primary production of mammalian lymphoid, myeloid and erythroid precursors, include the bone marrow, liver and spleen in fetuses, and the bone marrow in adults (Owen et al., 1977; Levitt and Cooper, 1980; Whitlock et al., 1985). Differentiation of the lymphoid population then begins in the bone marrow which produces pre-B and pre-T cells (LePault, 1983). Pre-T cells subsequently migrate to the thymus, which is the primary site of T cell development and maturation (Owen, 1972). The population of immature lymphocytes containing cytoplasmic immunoglobulin M (cIgM), but lacking detectable surface Ig, termed pre-B cells, have been found in livers from 11-12 day mouse, 7 week human and 21 day rabbit fetuses several days before surface IgM-positive B cells appear (Raff et al., 1976; Gathings et al., 1977; Andrew and Owen, 1978; Hayward et al., 1978). These and other studies have provided evidence that these cells are the precursors of B lymphocytes (Freitas et al., 1982; Whitlock et al., 1985).

In a normal, unmanipulated adult mouse, the bone marrow produces very large numbers of B lymphocytes. Although still debatable, it is believed that the bulk of B cell generation in the bone marrow is guided solely by internal driving forces and is independent of environmental influences. The rate of bone marrow B cell production is actually so high that within 2-4 days enough new B lymphocytes are produced to replenish the whole periphery of the mouse (Freitas et al., 1989; Osmond, 1986). These newly formed B cells are found to be immunocompetent, expressing surface Ig, and receptive to activation stimuli. These B cells subsequently migrate to the peripheral blood, lymph nodes and spleen. Upon further maturation, the cells become surface IgM+ / sIgD+, sensitive to antigen and mitogen

activation, and become the primary resting B-cell population in the adult spleen.

The B cell response. The entire sequence of events leading to a B cell response is generally categorized into three stages: activation, proliferation, and differentiation. These stages of B cell development have been viewed as sequential events, initiated and regulated by distinct and specific signals (Dutton and Swain, 1987; Jelinek and Lipsky, 1987). Classically, B cell responses induced by various stimuli have been characterized as either T cell independent (TI) or T cell dependent (TD). This characterization is based on their requirement for T cell factors to support the differentiation into antibody-forming cells. Today, this sequence of events is no longer so clearly defined. Recent studies suggest a variety of T cell derived lymphokines may potentially be involved in each step of the B cell response, including preparing small resting B cells to respond before interaction of surface Ig receptors (Oliver et al., 1985; Rabin et al., 1985). B cell responses may then vary only in the degree of T cell help required. Thus, each stage of the B cell response will be discussed in terms of the effects of the classically defined TI and TD antigens, rather than the categorical TI versus TD response.

B cell activation. The first step in initiation of the B cell response is the activation of the resting B cell. Activation is generally defined as the transition of G_0 resting cells to the G_i stage with subsequent entry into the cell cycle. For murine B cells this state is mediated by phophatidylinositol hydrolysis, diacylglycerol generation, and protein kinase C activation, associated with membrane depolarization, increased expression of I-A and

an accelerated entry of cells into S-phase upon exposure to the appropriate second signals (Crumpton et al., 1976; Walker et al., 1986; Coggeshall and Cambier, 1984; Clark and Lane, 1991). Activators, such as antigens, capable of inducing this transitional state in murine B cells are multiple. Classically, they have been divided into two categories, either T-independent (TI), or T-dependent (TD) antigens.

T-independent antigens. TI antigens in general are considered poorly metabolized, high molecular weight polymers made up of simple repeating units (Britton et al., 1968; Sela et al., 1972). These properties are considered important for their signalling mechanism which is believed to be via crosslinking of cell surface receptors. Studies comparing immunologically immature mice with normal adult mice of the CBA/N strain, which carries an X-linked B cell deficiency, led to the definition of two classes of T independent antigens. Type 1 TI antigens are capable of stimulating B cells carrying the defective trait and immature normal B cells, whereas type 2 TI antigens are only capable of stimulating normal mature B cells (Mosier et al., 1976; Mosier et al., 1977a). Examples of type 1 TI antigens include LPS (Moller and Michael, 1971; Kearney and Lawton, 1975), and Brucella abortus organisms (Mond et al., 1974). Examples of type 2 TI antigens include ficoll (Mosier et al., 1974), and dextran (Dorries et al., 1974).

Early studies demonstrated that certain B cell mitogens were capable of activating suitable target B cells directly in the absence of any accessory cells such as macrophages or T cells (Coutinho et al., 1974). However, as noted earlier, the role of T cell lymphokines is less clearly defined and may be necessary in activating B cells, challenging the notion that B cell

responsiveness occurs in a truly T-cell independent fashion for TI antigens (Endres et al., 1983; Mond et al., 1983). In both these studies, it was demonstrated that both type 1 and type 2 TI antigen induced responses could be eliminated by extensive depletion of T cells from the responding cell populations. Only type 1 TI antigens were able to elicit a very weak detectable response. Thus, it has been suggested it may be more accurate to reclassify type 1 and 2 TI antigens as T cell regulated type 1 and type 2 antigens since neither group is entirely T cell independent as previously categorized (Kenny et al., 1981; Mond et al., 1983; Pike and Nossal, 1984). This in turn would suggest that there is no such thing as a T-independent response, rather just degrees of difference between the amount of T cell help necessary for the culmination of a B cell response.

T-dependent antigens. T-dependent (TD) antigens have been defined as those antigens known to require T cell help to allow B cells to fully differentiate into antibody producing cells. Protein antigens were described as TD antigens because they failed to stimulate or only marginally stimulate an antibody response in athymic nu/nu mice (Jones, 1987). The basic nature of T cell help is the collaboration between a T cell, specific for a "carrier" epitope on an antigen, and a B cell specific for a "haptenic" epitope. The evidence for the need for a collaborative interaction between antigen-specific T and B cells in generating an antibody response to a TD antigen originated from studies demonstrating a requirement for "linked recognition" of antigen by both cells (Mitchison, 1971). This is described as the necessity of the hapten and carrier components of the antigen to be covalently linked in order for carrier specific T helper cells to cooperate with hapten-specific B cells.

The theory of linked recognition was further substantiated by studies demonstrating the TD antibody response was restricted by major histocompatability complex (MHC) encoded determinants ("self"-antigens) on the surface of the B cell (Katz et al., 1975; Sprent, 1978; Marrack and Kappler, 1980). It was shown that receptors on T cells must recognize "fragments" of processed antigen presented in the context of MHC-encoded class II (Ia) molecules on antigen-presenting cells (APC) (Kappler et al., 1981; Hedrick et al., 1982; Haskins et al., 1983). Originally, antigen processing and presentation for T cell recognition was thought to be mediated primarily by macrophages (Thomas and Shevach, 1976; Swierkosz et al., 1978). Macrophages have long been known to play an important role in both primary and secondary antibody responses (Pierce et al., 1972; Unanue, 1972). Macrophages are essential for promoting the viability and survival of lymphoid cells *in vitro*, through elaboration of factors, but also function in antigen processing and presentation. More recently though, several studies have demonstrated the ability of B cells to serve as antigen presenting cells (Chestnut and Grey, 1981; Tony and Parker, 1985; Lanzarecchia, 1985).

Thus, the minimal model for B cell activation by a TD antigen includes binding of antigen to surface membrane bound immunoglobulin and T cell help rendered by helper T cells that recognize processed antigen in context of class II MHC on the B cell surface. The result of this interaction is the elaboration of factors from T helper cells and/or macrophages which then induce the B cell to proliferate and ultimately to differentiate (Kishimoto, 1985; Swain, 1989). Additionally, T helper cells can be divided into two subsets called Th1 and Th2 based on their different profiles of lymphokine secretion (Mosmann et al., 1986; Cherwinski et al., 1987). Some of the

factors known to date to be involved in the early steps of B cell maturation, regardless of the specificity of the activation stimuli, include the following:

B cell growth factor (BCGF) was one of the first lymphokines to be described with an apparent specific action on B cells, of which 2 different molecular weight (MW) forms with distinct activities had been identified (Howard et al., 1982; Melchers et al., 1983; Corbel and Melchers, 1984). BCGF1 is now termed interleukin 4 (IL-4) and has been shown to act on both resting B cells, causing an increase in expression of Ia, and to enhance proliferation of already stimulated B cells (Roehm et al., 1984; Noelle et al., 1983). BCGFII, now termed IL-5, is not involved with B cell activation, rather it is described as a growth factor and a differentiation factor (Takatsu et al., 1980; Swain and Dutton, 1982; Swain et al., 1988). Both of these factors are secreted by the Th2 T helper cell subset.

IL-1 and IL-6, are two lymphokines produced by macrophages. It is postulated that these factors are released upon interaction between T cells and macrophages acting as antigen-presenting cells. IL-1 has been implicated in both T and B cell activation (Howard et al., 1983; Durum et al., 1985; Booth and Watson, 1984), while IL-6 is suggested to be involved in both T and B cell differentiation (Hirano et al., 1986; Garman et al., 1987).

IL-3 and GMCSF are other lymphokines produced by T helper cells which have been demonstrated to have strong colony stimulating activity as well as effects on differentiation of B cells (O'Garra et al., 1988; Clark-Lewis and Schrader, 1988). Some lymphokines from T helper cells have been shown to drive B cell proliferation and differentiation in the absence of other cells. In particular IL-2 and γ-interferon, produced by Th1 cells, can synergize in driving antigen primed B cells through to antibody production (Swain et al.,1981; Liebson et al., 1984). While IL-2 appears to be required

for optimum B cell responses, γ-interferon has been shown to display both suppressive and enhancing effects on proliferation and differentiation under certain conditions (Rabin et al., 1986; Snapper and Paul, 1987).

B cell maturation begins with the stem cell and proceeds through commitment to the B cell lineage as evidenced by expression of cytoplasmic IgM. Up to this point, maturation is antigen-independent. After expression of cytoplasmic, and subsequent surface immunoglobulin, maturation becomes an antigen-driven process. Resting B lymphocytes after sIg interaction with specific antigen are triggered to ultimately form antibody-secreting plasma cells or memory cells (Pike and Nossal, 1986).

Historically, several theories of significant contribution were put forth to explain the production of antibodies and diversity of the antibody repertoire. Probably the earliest theory was that of Erlich (1906) deemed the "side-chain" theory. Erlich claimed the antibody was a distinct molecular entity; a receptor, on the cell surface which possessed a discrete conformation that allowed for specific interaction with a complementary configuration on the antigen molecule. Next was the "antigen-template" theory proposed by Breinl and Haurowitz (1930), Mudd (1932), and Pauling (1940). The antibody was thought to be a rather pliable molecule which was molded into a complementary structure to the antigen. The antigen basically served as a template to mold preformed antibody molecules. Another theory termed the "modified-enzyme" theory posed that cellular uptake of antigen induced enzymes directed at destroying the antigen, and circulating antibodies simply were replicas of the enzymes lacking enzymatic activity (Burnet, 1941). In 1955, Jerne presented the "natural-selection" theory, later coined "clonal selection" (Jerne, 1955; Burnet, 1957), which was the first to suggest antibodies were produced continuously with a variety of unique

binding sites, and antigens simply selected the antibody of best fit, then once bound were able to be phagocytized by macrophages. Crucial to this theory is the fact that only one antibody specificity can be produced by a single B cell and its clonal progeny.

To date it is known that, in the antigen-independent phase of differentiation, changes occur which involve permanent genetic alterations. More specifically, these changes involve the rearrangements of both the heavy and light chain variable genes of the immunoglobulin molecules (Leder et al., 1974; Tonegawa et al., 1978; Davis et al., 1980; Early and Hood, 1981). In particular, each antibody consists of heavy (H) and light (L) polypeptide chains encoded by a combination of multiple, discontinuous germline gene segments.

The heavy chain variable region consists of variable (V_H), diversity (D_H), and joining (J_H) gene segments. The recombination of these regions occurs in an orderly manner, beginning with a D - J join which is then recombined with one of up to 1000 Vh gene segments (Sakano et al., 1980; Kurosawa and Tonegawa, 1982; Brodeur and Riblet, 1984; Pascual and Capra, 1991). Diversity of the antibody repertoire stems from several stages within the process of V region generation. First, in mice, one of >20 possible D gene segments joins to one of 6 J segments, which in turn combines with one of up to 1000 V genes for a possible 10^6 - 10^7 Vh regions generated just by different combinatorial associations (Klinman and Linton, 1988; Bangs et al., 1991).

Immunoglobulin light chains consist of a V_L and J_L gene segment (Leder et al., 1974; Brack and Tonegawa, 1977). The joining of these two gene segments results in the light chain variable region. Two unlinked loci (kappa and lambda) are able to produce light chains. Variation in the kappa

light chain also arises from combinations of four J genes with an estimated 90 - 320 V genes (Zeelon et al., 1981; Cory et al., 1981), while the lambda light chain is more restricted with a choice of two V genes able to join one or more J gene segments (Tonegawa et al., 1978; Miller et al., 1981).

Additional diversity is created in both the heavy and light chains during recombination of the V-D and D-J gene segments which may occur at varying nucleotide positions, and/or through nucleotide additions (Alt and Baltimore, 1982; Tonegawa, 1983; Heller et al., 1987). When this is coupled with the added diversity generated by the random combinations of heavy and light chains, it is calculated that more than 10^{10} different antibody molecules are possible (Berek and Milstein, 1988; Vitetta et al., 1991).

After functional recombination of heavy and light chain V regions, further heavy chain gene rearrangements are inhibited such that a single B cell and its progeny produce only one functional Vh and Vl variable domain (Alt et al., 1982; Weaver et al., 1985). Initially, a given Vh gene is first expressed in combination with the constant (Ch) region gene Cm. Slightly later, however, a B cell is capable of expressing both IgM and IgD membrane forms of heavy chain constant (C) region genes (Knapp et al., 1982). Together, the functional heavy chain and light chain comprise a complete immunoglobulin molecule of the IgM or IgD class.

B cell proliferation. Proliferation of B cell precursors provides a mechanism whereby a small number of antigen-specific or activated cells can be expanded to provide a more effective antibody response (Burnet, 1959). Though proliferation is considered necessary before resting cells may become antibody secreting cells, this is not a foregone conclusion. It remains a matter of controversy as to whether precursor cell division is

always a prerequisite. A number of studies have demonstrated evidence of maturation to antibody secretion occurring in the absence of cellular division (Andersson and Melchers, 1974; Melchers et al, 1980; Chen et al., 1981; Gross and Rucks, 1983).

Also debatable is the question as to whether proliferation continues after differentiation of B cells to antibody producing cells. There is recent evidence indicating cells synthesizing and secreting antibody are an actively proliferating population (Merrill and Ashman, 1980; Jelinek and Lipsky, 1983). They stated that after polyclonal stimulation *in vitro*, antibody producing cells are not terminally differentiated plasma cells, but rather an actively cycling antibody secreting population. It is suggested that proliferation of Ig secreting cells may play an important part in determining the magnitude of the response. A more recent study by Jelinek and Lipsky in 1985 explored the assumption that the initial proliferative phase is necessary to expand the number of Ig-secreting cell precursors. From their results they concluded that proliferation is necessary to induce responsiveness to differentiation, but not to increase the number of responsive precursors.

B cell differentiation. Since the time of Jerne's proposition that the antibody specificity of a given B cell is genetically determined, the relative contribution of evolutionary selective forces as opposed to random somatic events in shaping the expression of a mature antibody repertoire has been debated. Once a given antigen has 'selected' the best fitting antibody producing clone, this clone is expanded and triggered to further increase in affinity for this antigen (Siskind and Benacerraf, 1969; Julius and Herzenberg, 1974). Affinity maturation has been shown to be concomitant

with somatic mutation of Ig V regions (Berek et al., 1985; Wysocki et al., 1986), producing antibodies with increased specific affinity. As a high rate of somatic mutation is not detected early in the response, but increases with time (Kaartinen et al., 1983; McKean et al., 1984; Berek et al., 1985), the process appears to be antigen-driven (O'Brien et al., 1987). This appears to be particularly important for TD antigens and is heavily reliant upon T cell help.

Upon further differentiation these B cells may begin to synthesize other classes of antibodies. This is accomplished through a second genetic rearrangement known as a S-S recombination, or more generally as isotype switching (Honjo et al., 1981; Bergstedt-Lindqvist et al., 1984). This rearrangement occurs between switch (S) regions located 5' to each Ch gene, and combines the same Vh region with another Ch gene downstream. The five major classes of isotypes in mammals are designated as mu, delta, gamma, epsilon, and alpha, which can then be further divided into subisotypes for gamma (γ1, γ2a, γ2b, and γ3, in the murine system). Each of these isotypes differs not only at the nucleotide level, but functionally and physically as well. Because the different heavy chain constant regions possess distinct biological effector functions, isotype switching allows a single B cell clone to maintain antigenic specificity while altering its effector function (Vitetta et al., 1991). Isotype switching and the secondary or memory response have been shown to be highly T-dependent. It has been demonstrated that different activation stimuli will preferentially induce production of a particular isotype or subisotype of antibody (Perlmutter et al., 1978; Slack et al., 1980). Thus, upon activation by TI antigens, IgM, IgG3 and IgG2b are mainly secreted, for TD antigens; eventual switching to IgG1, IgG2, IgA, and IgE is usually induced.

The mechanism by which T cells influence isotype switching has been shown to be mediated through T cell lymphokines (Isakson et al., 1982; Coffman et al., 1988; Esser and Radbruch, 1990). Therefore, different lymphokines have been shown to regulate antibody-isotype expression. The major differentiation factors include a heterogeneous population under the heading B cell differentiation factors (BCDFs). The two major BCDFs induce IgM and IgG1 expression, although there is evidence for IgA and IgE specific BCDFs as well (Mayer et al., 1982; Ishizaka, 1984). Additionally various interleukins such as IL-4 and γ-interferon can influence isotype expression (Coffman et al., 1986; Snapper and Paul, 1987).

Salmonid B cell ontogeny. In the teleost, the sites of lymphohematopoeitic activity include the kidney, thymus and spleen. In addition to these organs, lymphocytes, including B cells, can be found in the peripheral blood, gut-associated lymphoid tissue (GALT), and in the lymphatic vessels that are differentiated from the vascular system (Pontius and Ambrosius, 1972; Zapata and Cooper, 1990).

During ontogenetical development, the thymus has been shown to precede the spleen and kidney in the appearance of lymphocytes, though hematopoeitic stem cells are already identifiable in the kidney well before the onset of lymphoid differentiation in the thymus (Ellis, 1977; Grace and Manning, 1980; Zapata, 1981). The thymus exists as a pair of lobes located on each side of the gill cavity (Chilmonczyk, 1983), and consists of thymocytes and lymphocytes in varying degrees of maturation, in addition to some erythrocytes and macrophages (Yasutake and Wales, 1983).

Though differentiated lymphocytes may first appear in the thymus, it is currently believed that the anterior kidney is the primary hematopoietic

organ in the teleost, and it has been compared to mammalian bone marrow in function (Zapata, 1979; Irwin and Kaattari, 1986; Zapata and Cooper, 1990). Every line of hematopoietic differentiation has been observed in the kidney including pluripotent stem cells, as well as immature and mature red and white blood cells (Smith et al., 1970; Zapata, 1979; Al-Adhami and Kunz, 1976). The kidney can be divided into two distinct segments; an anterior and a posterior segment, though the tissue structure is the same in both areas (Ellis and de Sousa, 1974; Zapata, 1979). The primary site of hematopoiesis is found in the anterior one-fourth of the kidney which contains lymphatic vessels and opens into the coelomic cavity.

Alternately, the spleen is suggested not to be essential for immunological maturation and, in most fish, serves as an accessory hematopoietic organ. This has been evidenced by the studies demonstrating that kidney and thymus lymphocytes display surface immunoglobulin and respond in a mixed lymphocyte reaction even when the spleen is present only in a rudimentary form (Ellis, 1977; Tatner and Manning, 1983; Yasutake and Wales, 1983). Additionally, the spleen when compared to the anterior kidney has been shown to harbor B cells comprised of a different repertoire than those found in the anterior kidney (Kaattari and Irwin,1985). Splenic lymphoid tissue is also not highly developed in teleosts. It differs from the mammalian spleen in that the red and white pulps are diffuse and connective tissue is not prominent (Robertson and Wexler, 1960; Anderson, 1974).

Salmonid B cell response. Phylogenetic studies generally agree that the earliest manifestations of immunoglobulins possessing antibody function are seen in fish (McKinney et al., 1977; Sima and Vetvicka, 1990). Initial

assessment of a teleost humoral immune response *in vitro* can probably be attributed to Smith et al. (1967). Using a modification of the hemolytic plaque assay, cells producing hemolytic antibody were found in two organs, the spleen and the pronephros in bluegill, *Lepomis macrochirus*. Using similar techniques, immunocompetent cells were later found in the spleen and anterior kidney of the rainbow trout *Oncorhynchus mykiss* (Chiller et al., 1969a; Chiller et al., 1969b; Pontius and Ambrosius, 1971). These studies demonstrated both antigen binding and antibody producing cells could be found in these organs. The ability to produce antibodies led to the assumption that these fish possessed cells equal in at least one function to the B lymphocytes of mammals. But early studies could not distinguish if B lymphocytes in fish were specialized and therefore separate from T cells (or cells with T-like functions) or if B lymphocytes were multifunctional and served in both capacities as B and T cell combined.

Much of the confusion resulted from studies where different methods were employed to separate B and T cells using polyclonal antisera raised against fish immunoglobulin. These antisera, being polyclonal, demonstrated crossreactivity between immunoglobulin and other lymphocyte surface markers, invariably reacting with all fish lymphocytes and thus was ultimately useless (Clem et al., 1991). In contrast, rosette formation and nylon wool filtration were used with some success to separate two distinct types of mitogen-reactive, antigen-binding cells (Cuchens and Clem, 1977; Ruben et al., 1977).

B cell activation. Preliminary evidence of the existence of two separate lymphocyte lineages comes from *in vitro* mitogenic studies in the teleost. Rainbow trout thymocytes have been shown to respond only to the T

cell mitogen, ConA, but not to the B cell mitogen, LPS, whereas anterior kidney lymphocytes responded to LPS only, and spleen and peripheral blood leukocytes responded to both mitogens (Etlinger et al., 1976a). Alternatively, Clem and coworkers found that bluegill cells were responsive to both ConA and LPS in the anterior kidney, and that lymphocytes in the thymus were capable of responding to LPS (Cuchens and Clem, 1977). Upon reexamination of rainbow trout organ distribution of mitogen responsiveness, Warr and Simon (1983), found the trout resembled the bluegill in that there was a clear response to both ConA and LPS by thymus, spleen, anterior kidney, and peripheral blood lymphocytes. While this suggested teleosts exhibit lymphocyte heterogeneity and organ distribution similar to higher vertebrates, the authors concluded that mitogenic studies alone were not sufficient to define true subpopulations, as subtle differences in technique or between species may be responsible for dissimilar results.

The somewhat ambiguous results from mitogen studies led to a more concentrated effort placed on defining B and T cell lineages by the virtue of the B cell's antibody response to TI or TD antigens. These methods employed the use of hapten-carrier conjugates, known to be either TI or TD antigens in mammals, to generate an anti-hapten PFC response *in vitro* from channel catfish leukocytes (Miller and Clem, 1984). *In vitro* primary and secondary PFC responses were distinguished using the above techniques, and further supported by evidence of primary *in vitro* activation in the rainbow trout with the same TI and TD antigens (Kaattari et al., 1986).

Examination of the primary *in vitro* activation with trinitrophenylated (TNP) haptenated antigens, allowed for the determination of the role of various carriers in the production of specific hapten antibodies. Activation by the classical TI antigen, TNP-LPS, is suggested to occur by providing a

concentrated mitogenic signal. The hapten, TNP, putatively targets and focuses the LPS mitogen onto the B cell surface by binding the TNP-specific receptors. In catfish it has been demonstrated that the response to TNP-LPS is independent of the sIg- population. Only the sIg+ and macrophage cell types were required (Miller et al., 1985).

The mechanism of activation by the classical TD antigen, TNP-KLH is still unclear. Miller et al. (1985), provided evidence indicating catfish PFC responses to TD antigens required three cell types; sIg+, sIg- and macrophages. Additionally, though the *in vitro* antibody response to TD antigens does occur without previous *in vivo* priming, the responses are lower if the fish are not previously exposed to the TD antigen. Further elucidation of the mechanism of TD activation in fish must await the clear separation and identification of T cells and their possible subpopulations.

Critical to the elucidation of two definitive lineages of lymphocytes (B and T) was the development of monoclonal antibodies (Mabs) specific for trout (DeLuca et al., 1983), carp (Secombes et al., 1983), and catfish (Lobb and Clem, 1982) immunoglobulin. In trout, the Mabs were used in panning techniques to separate Ig+ and Ig- populations, after which the population demonstrating Ig+ reactivity with the Mab responded to *in vitro* stimulation by LPS. However, though the correlation was clear, the LPS response could not be completely depleted from the sIg- population. The definitive nature of these results was clouded by the uncertain role of accessory cells in either population's mitogen responses.

B cell differentiation. In the catfish, lymphocyte separation studies using Mabs led to the consistent finding that monocytes/macrophages were required as accessory cells in the PFC response to hapten-carrier conjugates (Miller et al., 1985). Further investigations were focussed on

whether accessory cell function could be attributed to a soluble factor produced from the macrophage, similar to IL-1 in mammals (Clem et al., 1985; Miller et al., 1985). It was discovered that supernatants from peripheral blood monocytes stimulated by LPS could replace the requirement for accessory cells in mitogen, as well as TI and TD PFC responses (Clem et al.,1985). The molecule(s) responsible for this activity, appear to be two catfish IL-1 like molecules differing in MW, and cross-reactivity with mammalian IL-1 (Ellsaesser, 1989). This observation has raised the question as to the necessity of antigen processing and/or presentation by the macrophage in a TD response in catfish. Studies addressing this issue are underway and it may be that as in mammals (Chestnut et al., 1982), B cells as well as macrophages are involved in antigen processing and presentation to T cells.

The antibody molecule produced by various teleost species is predominantly a form resembling IgM of higher vertebrates, except that it is primarily seen as a tetramer (Bradshaw et al., 1969; Shelton and Smith, 1970; Acton et al., 1971; Marchalonis, 1977). Additionally it appears some fish possess monomeric forms of this IgM molecule; whereas all fish appear to possess the higher molecular weight forms whether it be as dimers, tetramers, pentamers, or hexamers (Warr and Marchalonis, 1982; Lobb and Clem, 1983). It has been proposed that the monomeric forms are present in all fish species, but in varying concentrations.

Analysis of purified chum salmon IgM by Kobayashi et al. (1982), demonstrated a tetrameric structure of a MW of 730,000. A subunit SDS-PAGE analysis revealed a two-chain structure, reportedly heavy and light chains, whose MW was calculated to be 72,000 and 23,000 respectively. The amino acid composition of the light chain purified from chum salmon

IgM is similar to those reported for the paddlefish, gar and catfish (Acton et al., 1971b). The heavy chain amino acid composition was also found to be similar to those of the above mentioned fish. Interestingly, both PAGE and immunological testing failed to demonstrate the presence of a J chain, which is also absent in the gar and paddlefish, whereas the catfish have been reported to possess a J chain (Weinheimer et al., 1971).

More recently, Lobb (1986), has found that two distinct light chain isotypes, named F and G, exist in channel catfish. Examination of the expression of these light chains during an antibody response showed early antibodies contained 20% of G light chains, while antibodies produced later in the response consist of 90% F light chains. These results were the first to suggest that teleosts, as with higher vertebrates, possess different immunoglobulin isotypes. This was subsequently followed by reports of distinct heavy chain isotypes identified by Mabs prepared against channel catfish immunoglobulin to identify different populations of the approximately 700,000 MW tetrameric antibody. Immunoprecipitation and peptide mapping analyses using these three Mabs in various combinations demonstrated the catfish immunoglobulin identified by each Mab was antigenically distinct. Also, each Ig population contained both classes of catfish light chains (F and G) (Lobb and Olson, 1988). Additionally, levels of the three heavy chain isotypes were monitored during a response to the dinitrophenyl hapten. Results indicated one H chain isotype was preferentially expressed early and remained the predominantly expressed isotype during the response.

It is known that fish generally demonstrate a very limited repertoire of antibody specificities (Wetzel and Charlemagne, 1985; Espelid et al., 1987), and that antibody production is under complex genetic control as

immunoglobulins are expressed from a limited number of germline V genes (Cossarini-Dunier et al., 1986). It has been demonstrated that even partial inbreeding significantly reduces available antibody repertoires of fish. Heavy chain spectrotypes were compared between normal trout, self-fertilized and gynogenetic fish, and it was observed that the spectrotypes of self-fertilized trout were less heterogeneous than in the other two groups. In the past few years intensive research has been underway to study the immunoglobulin gene organization in fish. The majority of information has come from the study of two phylogenetically distant fish species, the horned shark, *Heterodontus francisci,* and the channel catfish, *Ictalurus punctarus.*

Kokubu et al. (1987) investigated the immunoglobulin heavy chain gene organization in the phylogenetically primitive elasmobranch, the horned shark. It was discovered that these fish have a unique organization of their immunoglobulin genes when compared to the mammalian organization. It appears that V_H-D_H-J_H segments are closely associated with individual constant region genes that can differ at the amino acid sequence. These segments are linked closely in multiple individual clusters, and D_H as well as junctional and N-type diversity account for the limited Ig variability (Kokubu et al., 1988a,b). Similarly, Harding et al. (1990), demonstrated a 'clustered' immunoglobulin gene organization in another primitive cartilaginous fish, *Raja erinacea.* The IgM-like heavy chain possessed a nucleotide sequence of 61-91% homology with that of the horned shark. Evidence was also presented for the presence of a second isotype in the *Raja erinacea.* Additionally, two light chain genes were cloned and sequenced in the shark whose sequence comparisons are consistent with mammalian-like framework and complementarity determinant regions. Amino acid sequences for the shark light chains seem

more related to mammalian lambda than kappa light chains (Shamblott and Litman, 1989).

Immunoglobulin gene organization was also investigated in the channel catfish. Sequence analysis confirmed that the catfish heavy chain was definitely similar to chicken and mouse mu chains (Ghaffari and Lobb, 1989a). Upon completion of the heavy chain mRNA sequence analysis of the catfish (Ghaffari and Lobb, 1989b), phylogenetic sequence comparisons of the catfish C region domains indicated the C_H1 and C_H4 domains were the most highly conserved. Gene titration experiments also established that the catfish C_H gene is represented by a single genomic copy. These results indicated that the genomic organization of H chain genes in the catfish must be different from that defined in sharks, and more closely resembles the mammalian organization. Sharks possess multiple C_H genes, as one is represented in each of their multiple clusters, while mammals have a single C_H gene region. As is characteristic for higher vertebrate heavy chains, catfish have been shown to possess different V_H families. It is likely that a V_H gene undergoes functional recombination with putative D_H gene segments and one of several J_H segments similar to higher vertebrates. Based upon sequence relationships and hybridization analyses, five different groups of V_H genes have been identified which may be classified as V_H families. Genomic southern blots have estimated >100 possible different germline V_H genes (Ghaffari and Lobb, 1991). In support of these results, Amemiya and Litman (1990), examined the immunoglobulin heavy chain V region locus in another teleost, the phylogenetically primitive *Elops saurus*. Genomic analysis using C_H, J_H and V_H specific probes demonstrated the presence of only a single hybridizing C_H gene and several J_H elements. Evidence has been presented that the Ig V_H locus in

this primitive teleost is more comparable to that seen in mammals than that seen in the sharks.

Mammalian suppressor systems in immunoregulation. Mammalian suppressor cell subpopulations are numerous and heterogeneous, both phenotypically and functionally. Suppressor cells may be induced after exposure to antigens, such that they are antigen-specific, or they may be found present in a naive animal. In the latter case, the natural immune system has the capacity to initiate host defense mechanisms that do not appear to require sensitization, thus these cell mediators are not antigen-specific and are not MHC-restricted. Other components of the immune system respond to foreign materials in an antigen-specific manner, and generally exhibit a memory response upon reexposure to the antigen (Tilden and Clement, 1989).

Natural effector cells. Although it has been established that the cells comprising the natural immune system are derived from bone marrow precursors, these cells are sufficiently diverse morphologically, phenotypically, and functionally to make it difficult to precisely distinguish or define their unique characteristics. To date, the major function of these cells is their suppressive capacity. Natural effector cells suppress the proliferation of a broad variety of cell types, although enhancement of certain responses has also been shown for these cells through the elaboration of stimulatory lymphokines.

As a group, the characteristic morphology of these natural effector cells is defined by the presence of lysosomes dispersed throughout the cytoplasm, phenotypically identifying them as large granular lymphocytes

(LGL) in mammals (Grossi et al., 1978; Saksela et al., 1979; Grossi et al., 1982). More importantly, they are not antigen-specific, nor MHC-restricted in their immunoregulatory function. They also appear to be nonadherent to nylon-wool or plastic, lack surface immunoglobulin, and for the most part, assume a null phenotype; expressing no surface antigens associated with mature lymphoid or myeloid lineage cells (Dorshkind and Rosse, 1982; Holda et al., 1986; Tilden and Clement, 1989; Reynolds and Ortaldo, 1990).

Some of the members in the family of large granular lymphocyte effector cells include natural killer (NK) cells, natural cytotoxic (NC) cells, lymphokine activated killer (LAK) cells and natural suppressor (NS) cells (Maier et al., 1986; Reynolds and Ortaldo, 1990). Although it appears these populations are closely related to one another, much work has revealed the heterogeneity and complexity of these natural effector cells, both in regards to function and interaction with other cell populations. Though all members of this family demonstrate suppressor function, the mechanism by which these activated effector cells mediate their immunoregulatory function is still controversial, and may vary between cell types. For example, NK and NC cells are known to possess cytolytic activity (Phillips and Lanier, 1986; Rowley and Shah, 1986), while NS cells are believed to secrete soluble factors that mediate their suppressive activity (Bassett et al., 1977; Pavia and Stites, 1979; Argyris, 1981; Atkinson et al., 1986).

Much work has been devoted to characterization of individual natural effector cell populations. Natural killer cells were originally described as effector cells capable of *in vitro* lysis of specific tumor cell targets (Herberman et al., 1979; Herberman, 1982). NK activity is defined by the selective lysis of particular target cells (classically the YAC-1 cell line in the murine system) in the absence of prior priming or specific memory. NK cells

possess C3bi and Fcg receptors, but may vary in their expression of other cell surface markers (Lanier et al., 1986). There also appears to be a direct relationship between ADCC activity and FcgR expression. Additionally, it was demonstrated that these cells played a part in the regulation of the growth and differentiation of hematopoietic systems (Lotsova, 1986; Kiessling et al., 1977; Cudkowicz and Hochman, 1979; Hansson et al., 1982). Both *in vivo* and *in vitro*, NK cells were shown to inhibit allogeneic as well as autologous granulocyte-macrophage colony forming units (CFU-GM).

There is also evidence of NK regulation of antibody responses. A cloned human NK cell line was shown to lyse LPS-activated B cell blasts, and suppress LPS-induced antibody production (Nable et al., 1981; Nable et al., 1982). Purified NK cells have also been shown to suppress pokeweed mitogen (PWM)-induced antibody production after activation with immune complexes (Tilden et al., 1983), or without prior activation (Arai et al., 1983). NK cells have also been suggested to control the proliferation and differentiation of thymocytes (Ono et al., 1977; Hansson et al., 1979). Thus it has been postulated that NK cells may interfere with B cell activity indirectly through the inhibition of T cell help (Kumagai et al., 1989). There is, therefore, the potential for NK regulation of antibody responses at multiple points during B cell maturation and a role for NK cells in the regulation of the growth and differentiation of hematopoietic and lymphoid cells. Interestingly, though NK cells originate and differentiate in the bone marrow, active mature NK cells are almost entirely absent from the bone marrow of healthy human donors (Haller and Wigzel, 1977; Perussia et al., 1983). The proportion of NK cells generally is highest in the blood, spleen and liver, but low in bone marrow and thymus (Kiessling and Wigzel, 1981). Experimental

and clinical observations support the hypothesis that NK cells are the cellular effectors for certain types of pathological dysregulation of hematopoiesis. However, the evidence for such a role in maintaining hematopoietic homeostasis is much less compelling (Trinchieri et al., 1989).

Natural cytotoxic (NC) cells are similar to NK cells in their lytic activity against certain tumor targets *in vitro* (Stutman and Cuttito, 1981), but differ in their target specificity for transformed cells that form solid tumors (Lin et al., 1983). NC activity generally requires longer periods of incubation when compared to NK activity, up to 18 hours or more, and in the mouse the target cell used to identify NC activity is the WEHI-164 tumor cell. Also NC activity appears earlier in development than does NK activity. In the human, contrary to the mouse, NC activity has not been definitively associated with any one cell type. It has been postulated that NC activity may not pertain to a specific cell type, but rather may be a function possessed by many cell types (Reynolds and Ortaldo, 1990).

Another member of the LGL family are the natural suppressor (NS) cells. NS cells, like NK and NC cells are of the 'null' phenotype, and have suppressor activity which is antigen-nonspecific and MHC-nonrestricted (Maier et al., 1986). Mature or activated NS cells also appear to be resistant to irradiation (Holda et al., 1990). The primary difference between NS cells and NK or NC cells is that NS cells do not function through cytolysis, and do not kill NK specific targets (Duwe and Singhal, 1979; Corvese et al., 1980; Okada and Strober, 1982; Oseroff et al., 1984). Natural suppressor activity is defined by these cell's ability to suppress immunogenic or mitogenic lymphocyte responses. The most common assay of NS activity is to measure the inhibition of proliferation as in MLR, tumor cell proliferation, and mitogen cultures (Strober, 1984; Maier and Holda, 1987; Maier et al., 1989;

Sugiura et al., 1990). Studies examining the mechanism by which NS cells mediate their suppression postulate that NS cells may act through secretion of soluble inhibitory factors (Atkinson et al., 1986; Hertel-Wulff and Strober, 1988). NS cells have been demonstrated to suppress immune responses at low suppressor-to-target ratios (below 1:1), and both adult BM and neonatal spleen cell culture supernatants were shown to inhibit targets in a similar manner to their whole cell counterparts. More specifically, studies have provided evidence that the soluble factor produced by NS cells blocks IL-2 production and activity, or may inhibit IL-2 receptor expression (Maes et al., 1988; Maier et al., 1989).

NS activity is also assessed by the inhibition of antibody production. In the initial studies, prior to the isolation of purified natural suppressor cells, investigators observed the inhibition of *in vitro* antibody production by a suppressor cell in normal murine and human bone marrow (Duwe and Singhal, 1979; Corvese et al., 1980; Bains et al., 1982). Suppression of both TD and TI spleen cell antibody responses was observed without apparent cytotoxicity in the murine studies, while suppression of spleen, tonsillar, and autologous peripheral blood cell PFC responses was observed in the human system. Interestingly, while a 1:10 ratio of BM cells to SP cells resulted in suppression in the mouse, a ratio of 1:1 or greater was used for the human inhibition studies. In a more recent study, Mortari et al. (1986) extended his coworkers' earlier studies of human BM suppressor cells and through cell separation techniques provided evidence suggesting NS cells may prevent the induction of autologous B cell primary IgM antibody responses within the bone marrow compartment.

Another important characteristic of NS cells is their location. NS cells normally can be found in neonatal lymphoid tissue (Rodriguez et al., 1979;

Okada and Strober, 1982) and in adult bone marrow (Duwe and Singhal, 1979; Dorshkind and Rosse, 1982). Alternatively, NS activity is not usually seen in normal spleen, but does arise after either total lymphoid irradiation (TLI) (May et al., 1983; Weigensberg et al., 1984; Oseroff et al., 1984), after the induction of chronic graft-versus-host disease (GVHD) (Maier et al., 1985), or cyclophosphamide treatment (Greely et al., 1985; Segre et al., 1985). The importance of these locations of NS activity is two-fold. One is that they are all sites of considerable hematopoiesis. Neonatal spleen and liver as well as adult bone marrow are primary environments of hematopoiesis. For both TLI and GVHD lymphoid tissue is damaged and NS activity is observed upon repopulation during recovery. Secondly, these are all environments where tolerance can and does occur (Billingham et al., 1953; Storb, 1983; Strober, 1984). This may suggest a role for NS cells in hematopoietic regulation or proliferation, perhaps controlling cell growth through inhibition of growth factor production (Soderberg, 1985; Maier et al., 1986).

Another cell type demonstrating suppressive capabilities, not of the LGL family, are the macrophages (Ju and Dorf, 1985; Nelson, 1987; Schaefer et al., 1985; Wiltrout and Varesio, 1990). Macrophages are similar to LGLs being antigen-nonspecific and MHC-unrestricted in their suppression, but differ from LGLs phenotypically and functionally. Macrophages are esterase-positive, adherent, and express the MAC-1 and Ia antigens, which indicate LGLs are not mature macrophages, but several studies support the concept that NS cells may be related to the macrophage lineage somehow (Piguet et al., 1981; Snyder et al., 1982; Peeler et al., 1983; Kato et al., 1985). Results of these studies suggest NS cells may be an early less differentiated stage of the macrophage lineage.

Macrophages have been recognized for their roles in many forms of immunoregulation (Rosenstreich, 1981). They stimulate the natural immune system by generating a variety of important upregulatory compounds necessary for both antigen-nonspecific and antigen specific immune responses (Nathan, 1987), and also are able to produce toxic molecules to defend against foreign microorganisms (Somers et al., 1986). Additionally, macrophages have down-regulatory functions, which seem to be induced by some of the same stimuli that activate cytotocidal macrophages (Varesio, 1983; Nelson, 1987). Interestingly, Mishell and Dutton (1967), probably provided the first evidence of the suppressive activity of macrophages when they observed that addition of high numbers of macrophages inhibited antibody production in vitro. Since then, macrophages have been shown to be more inclined to suppress T-cell mediated responses or T-cell dependent responses than strict B cell responses (Nelson, 1987; Wiltrout and Varesio, 1990). These observations come from the major source of evidence for macrophage suppression; studies of tumor-bearing hosts. Suppressor macrophages are primarily identified in the spleens of these animals, which demonstrate depressed proliferation to T cell mitogens, decreased MLR, and suppressed T cytotoxic responses (Kirchner et al., 1974, 1975; Whitney et al., 1977). Thus the effect of macrophages on B cell responses and antibody production is less well known. The mechanism by which macrophages induce suppression is also not fully known. There are some reports indicating that prostaglandins and/or oxygen metabolites produced by the macrophage mediate the suppression (Metzger et al., 1980).

T suppressor cells. The last set of suppressor cells known to play an integral part in the regulation of the immune response are the T suppressor

cells. Suppressor T cells are capable of modulating both humoral (Rohrer et al., 1979; Sherr et al., 1984; Suemura et al., 1983) and cellular immunity (Bach et al., 1978; Asherson and Zembala, 1974; Greene et al., 1982), and are known to be responsible for phenomena associated with tolerance (Basten et al., 1975; Weigle et al., 1975). T suppressor cells have also been shown to be an important part of homeostasis which controls the course and magnitude of antigen-specific immune responses (Dorf and Benacerraf, 1984). Antigen-specific suppression has been demonstrated in most mammalian species investigated to date including mouse (Gershon and Kondo, 1970; Tada et al., 1976), rat (Schoen et al., 1982), monkey (Kontiainen et al., 1979) and human (Chaouat, 1978).

In the murine system, at least three T cell subsets are involved in the suppressor pathway (Weiner et al., 1990; Damle and Engleman, 1990). These include the Ts1 cells which function as suppressor-inducers upon interaction with antigen and class II MHC determinants on antigen-presenting cells. Ts1 cells then stimulate Ts2 cells, which in turn act as intermediaries or suppressor-amplifier cells that appear to trigger a third set, Ts3 cells, which function as the suppressor-effectors (Germain and Benaceraff, 1981; Green et al., 1983; Dorf and Benaceraff, 1984; Asherson et al., 1986). Upon interaction with antigen, these T cell subsets each produce specific factors that provide the necessary signals for the induction, activation and effector functions required for suppression. Though Ts1 suppressor-inducers require an antigen-specific trigger, the mechanism by which the final Ts3 suppressor-effector cells mediate suppression is still contraversial as some evidence demonstrates an antigen-nonspecific and genetically unrestricted mode of suppression (Greene et al., 1982a,b). These studies proposed that nonspecific factors produced by Ts3 cells

directly influence T helper cells to induce suppression of both cellular and antibody responses. Alternately, Dorf and coworkers have provided data suggesting that both Ts2 and Ts3 factors display dual genetic restrictions for both MHC and IgH linked genes in order to suppress the contact sensitivity reaction, but may not be IgH restricted in suppressing antibody responses. Thus, in the PFC system examined, TsF3 may act directly or indirectly on B-cell targets in suppression of the antibody response (Okuda et al., 1981; Sherr and Dorf, 1982; Sherr et al., 1983; Sherr et al., 1984).

Although there are a variety of cell populations capable of antigen-nonspecific, MHC unrestricted suppression of the immune system, only the T cell suppressor population appears capable of mounting an antigen-specific response leading to the elimination of a specific antigen or foreign substance. In their capacity as immunoregulatory cells, it has been demonstrated that some populations of bone marrow-derived B cell precursors cannot be rendered tolerant to antigen unless certain subpopulations of T cells are present (Gershon and Kondo, 1971; Okumura and Tada,1971; Claman and Miller, 1976). In keeping with the initial definition in division between the natural immune system and the specific immune system, it is important to point out that the T cell suppressor population is part of the specific immune system that requires quite specific antigenic stimuli in order to induce suppression. This classification then contrasts with the family of LGLs discussed earlier, which as part of the natural immune system, mediate suppression in an antigen-nonspecific and MHC-unrestricted manner.

Salmonid suppressor systems in immunoregulation. In contrast to the knowledge of T suppressor systems in the endothermic vertebrates, the

existence of functionally different T cell subpopulations, such as suppressor, cytotoxic, and helper cells in fish, has not been definitively demonstrated (Clem et al., 1991). There are some studies, however, which indirectly infer suppressor and helper functions may be separable in fish. One study using carp, demonstrated that intravenous injection of high doses of deaggregated soluble antigen could result in antigen-specific suppression of antibody formation (Serero and Avtalion, 1978). They were not able to rule out that this may have been the result of antigen-induced tolerance of either T or B cells, although it could be possible that antigen specific suppressor cells were involved.

Another study involving adult thymectomy in rainbow trout showed that thymectomized fish developed higher antibody titers following in vivo immunization when compared to sham-immunized controls. The authors speculated that these results may be due to the removal of a source of suppressor cells by thymectomy (Manning et al., 1982). Further support regarding the presence of suppressor cells in trout was derived from the findings that low (100-600 rads) doses of gamma-irradiation augmented the in vitro PFC responses to TNP-LPS and TNP-KLH (Kaattari et al., 1986).

Unpublished results indicating that this radiosensitive suppressor cell population actually was a subset of the T cell lineage was obtained with channel catfish (Miller et al., 1989). The results, though inconsistent, indicated that cultures containing gamma-irradiated T cells and unirradiated B cells can exhibit greatly enhanced responses when compared to unirradiated T and B cell mixes or unfractionated controls. Even though the above studies are preliminary, it seems probable that fish can have T cells with radiosensitive suppressor function similar to that seen in mammals. It may also be postulated that these suppressor populations play some role in

immunoregulation due to the evidence of their natural suppressive activity regarding the B cell antibody response.

There has been more research completed in the study of nonspecific cytotoxic cells in fish. Hinuma et al. (1980) is cited as the first to examine nonspecific cytotoxic reactions in fish. Fourteen species of fresh water fish were tested against xenogeneic target cells and they discovered cytotoxic cells could be found primarily in the kidney, though sometimes in the peripheral blood. Due to the wide variety of targets the cytotoxic cells exhibited activity against, it was assumed the reaction was not due to antigen-specific thymocytes, but rather that the effectors were more similar to mammalian natural killer cells. In contrast, another study suggested fish killer cells appear to be more similar to mammalian macrophages than NK cells of birds or mammals (Hibbs et al., 1972).

In later studies, Evans and coworkers (1984a), defined these cells as 'non-specific cytotoxic cells' and proposed that these were the evolutionary precursor to mammalian NK cells. They demonstrated these cells were a highly active population, found in the head kidney, spleen and peripheral blood in catfish (Graves et al., 1984). Additionally it was shown that lysis was dependent upon cell-cell contact (Evans et al., 1984a), and the NCCs were found to be non-adherent and non-phagocytic. Interestingly, catfish serum suppressed this NCC activity (Evans et al., 1984b).

Though the majority of studies were focused on cytotoxicity towards mammalian cell lines only, cytotoxicity directed against teleost cell lines has also been described (Moody et al., 1985). Four different teleost cell lines were found susceptible to lysis by kidney, spleen and blood leukocytes of *Salmo salar*, *Salmo gairdneri*, and *Notemigonus crysoleucas*. Cytotoxic activity was similar for all leukocyte sources tested, and it appeared that cell-

cell contact was required. Infectious pancreatic necrosis virus (IPNV) infected targets were also more readily lysed than noninfected targets, suggesting a role for NCC in the defense mechanism of fish, although the precise lytic mechanism of fish NCC is still unknown. In mammals, NK cells have been determined to function primarily as an important regulatory mechanism of immune responses, in addition to providing a significant barrier in natural resistance to diseases (Herbeman, 1981). Though data from NCC studies in fish may suggest a similar role in natural resistance to infection (Moody et al., 1985), the role as regulatory cells in the immune response has not yet been demonstrated.

CHAPTER 2

Mitogenesis of Rainbow Trout Peripheral Blood Lymphocytes Requires Homologous Plasma for Optimal Responsiveness

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SUMMARY

An improved method for obtaining optimal mitogenic responses in peripheral blood lymphocytes has been devised by utilizing autologous or homologous rainbow trout plasma. The use of 10% plasma in culture results in up to a sixty-fold increase in the proliferative potential of the peripheral blood lymphocyte response to lipopolysaccharide when compared to the more routinely used fetal bovine serum. Furthermore, it has been observed that lymphocytes which were unresponsive to *in vitro* mitogenic challenge when cultured in fetal bovine serum, responded well when cultured in the presence of trout plasma. Also in contrast to previous mitogen studies where maximal stimulation was reported to occur on day 4-5 of culture, the stimulatory effects of LPS were greatest on day 10 when plasma was employed. Together these data suggest that former conditions of lymphocyte cell culture, employing only fetal calf serum, not only fail to provide the optimal conditions for cell growth, but in many cases the essential conditions. However, these requirements are met by supplementation with trout plasma, which appears to contain heat stable factors responsible for the enhanced mitogenic responsiveness.

INTRODUCTION

Traditionally, the sera and media used in fish cell culture have been adapted from mammalian cell culture procedures with little or no modification (Wolf and Mann, 1980). Thus, fetal bovine serum has been used routinely in fish culture as the preferred serum supplement (Nicholson, 1989), though some groups have experimented with alternatives (Cuchens and Clem, 1977; Clem et al., 1981; Rosenberg-Wiser and Avtalion, 1982; Caspi et al., 1984; Faulmann et al., 1983). Determination of optimal conditions for catfish and bluegill lymphocyte culture included the testing of human, calf, rabbit, alligator, bass, grouper, bream (a collective term for all Lepomis species) and catfish sera or plasma (Cuchens and Clem, 1977). Bluegill lymphocytes responded best to bream plasma, while Faulmann, et al., observed little effectiveness in catfish lymphocytes unless a combination of 10% human plasma and 5% channel catfish sera was employed. In addition, they found fetal calf serum to be inhibitory when combined with either human or catfish serum. Avtalion and his coworkers (Rosenberg-Wiser and Avtalion, 1982; Caspi et al., 1984) examined rat, rabbit, horse, calf, human, dog, chicken and carp sera with various manipulations and determined that 2-4% charcoal-absorbed carp serum was optimal.

The majority of studies employing rainbow trout lymphocytes have been limited to the use of fetal bovine serum (FBS) as a supplement. Aside from FBS, only human serum and homologous rainbow trout serum have been tested for their ability to serve as alternative medium supplements (Tillit et al., 1988; Etlinger et al., 1976a). Utilization of FBS in lymphocyte culture has yielded varying results for both mammals and fish. Several reports have described results ranging from suppression (Cooperband, et al., 1968;

Etlinger et al., 1976a) to activation of mitogen responses (Sebesin, 1965; Etlinger et al., 1976b). A stimulatory effect of FBS in the absence of any mitogens has also been observed in peripheral blood lymphocyte (PBL) cultures from some individual fish (Etlinger et al., 1976b; Tillit et al., 1988).

Our studies reveal that FBS does not allow for maximal proliferation of lymphocytes in response to mitogens. Trout plasma supplementation on the other hand yielded consistently higher mitogenic responses. These data suggest that conclusions based on past functional studies which utilized FBS may be fundamentally incorrect. Thus, studies devoted to the determination of lymphocyte functional heterogeneity wherein non-responsiveness was observed, may be a result of suboptimal culture conditions rather than an absence of responsive cells (Rosenberg-Wiser and Avtalion, 1982; Tillit, et al., 1988).

MATERIALS AND METHODS

Animals. Shasta strain rainbow trout (*Oncorhynchus mykiss*) were obtained from Dr. Jerry Hendricks, Department of Food Science and Technology, Oregon State University, and were maintained at the Salmon Disease Laboratory in Corvallis, OR. This facility receives fish pathogen-free water at a constant temperature of 12°C. Fish were fed Oregon Moist Pellet (OMP) commercial salmon food daily.

Culture media. Lymphocytes were cultured in either tissue culture medium (TCM) consisting of RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% defined Fetal Bovine Serum, lot # 1111889 (Hyclone), or 10% trout plasma, and 0.05 ug/ml gentamycin sulfate (Whittaker Bioproducts Inc.). Plasma was obtained by diluting heparinized blood samples 1:4 with RPMI (containing no anticoagulant), resulting in a stock plasma concentration of approximately 25%. Final plasma concentrations were obtained by further appropriate dilutions in RPMI. Other serum sources included horse serum (HS) obtained from M.A. Bioproducts (Walkersville, MD). Rabbit serum was obtained from New Zealand White Rabbits. Blood was allowed to clot, serum removed, filter sterilized and stored at -20°C. Trout serum was obtained from caudal bleeds of anesthetized trout. Whole blood was allowed to clot overnight at 4°C, the serum was then separated from the blood cells, filter sterilized and stored at -20°C.

Mitogens. Stock solutions of lipopolysaccharide (LPS) from *E. coli* 055.B5 (Sigma Chemical Company, St. Louis, MO) were pasteurized by incubation in a 70°C water bath for 1 hour. Concanavalin A (Con A) and

phytohemagglutinin (PHA-P) were also obtained from Sigma, diluted in RPMI and sterilized by filtration through a 0.45 um filter.

Cell preparation and tissue culture. Peripheral blood leukocytes and plasma were obtained from trout anesthetized in benzocaine (ethyl p-aminobenzoate, Sigma) (Kaattari and Irwin, 1985). Caudal bleeds were taken in 10 ml sodium heparin Vacutainer tubes (Becton Dickinson and Co., Rutherford, NJ.). The blood was subsequently diluted 1:4 in RPMI-1640 and centrifuged at 17°C in 15 ml conical polystyrene tubes (Corning Glass Works, Corning, NY) for 10 minutes at 500 x g. The supernatant was removed and stored at 4°C as stock plasma. Blood cells were then resuspended in the original volume of RPMI-1640 and layered on an equivalent volume of Histopaque-Ficoll 1077 (Sigma). The tubes were centrifuged at 17°C for 45 minutes at 500 x g. The interface layer of cells between the RPMI and Histopaque was recovered and washed twice in 10 ml RPMI at 500 x g for 10 min, at 17°C. Cells were then resuspended in 5 ml RPMI and viability determined by trypan blue dye exclusion.

After enumeration, cells were resuspended to a concentration of 1 x 10^7 cells/ml in either TCM or RPMI with 10% plasma. Aliquots of 100 ul were cultured in individual wells of a 96 well flat bottom tissue culture plate (Corning Glass Works, Corning, NY). Mitogens in appropriate concentrations were then added in 10 ul volumes. Cells were incubated in an incubator culture chamber (Model 624, C.B.S Scientific Co., Del Mar, CA) at 17°C, in a blood-gas environment containing 10% CO_2, 10% O_2, 80% N_2. Cells were fed every third day with 25 ul of the appropriate medium.

Thymidine incorporation. Twenty hours prior to harvest, 10 ul (1 uCi) of 3H-thymidine (ICN Biomedicals, Inc., Irvine, CA) in RPMI was added to

each well. Cells were harvested as previously described by Kaattari and Yui (1987).

Heat treatment. The effect of temperature on the supportive function of plasma was determined by incubating 10% plasma at 37, 56, or 100°C for thirty minutes. Each heat treated plasma was then stored at 4°C until used in culture.

RESULTS

Various serum and plasma sources. Various sources of serum were tested either alone or in combination with homologous rainbow trout plasma in initial attempts to determine the optimal culture supplement. Horse, rabbit, and fetal bovine serum were examined for their effects on mitogenesis of rainbow trout peripheral blood lymphocytes. Additionally, each of these sera was used in combination with autologous plasma at the same concentration. Rainbow trout serum was also tested for its effects on mitogenesis. The data indicated that horse, rabbit, and fetal bovine serum were found to support minimal responses similar to those previously reported (Fig. 2.1A). However, autologous plasma either alone or in combination with any of the sera, provided conditions eliciting significantly higher proliferation (Fig. 2.1B). As can be seen in this experiment, cells which are nonresponsive to LPS when cultured in FBS, can respond when autologous plasma is added. When rainbow trout sera were employed, variable results were observed, from stimulation comparable to that seen with plasma, to suppression of mitogenic responses (Fig 2.1C).

Effect of plasma concentration. The effects of plasma concentration on the response of lymphocytes to LPS are shown in figure 2.2. The dose of LPS previously determined to be optimal for culture in FBS was used initially to determine the optimal plasma concentration for supplementation. Figure 2.2 is a representative study chosen from experiments involving eight individual fish. Cultures utilizing fetal bovine serum as the supplement resulted in little proliferation either with or without LPS (Fig. 2.2A). When cells were cultured in the presence of autologous plasma, a marked increase in proliferation of LPS responsive cells was observed (Fig. 2.2B).

Plasma doses from 25% to 1% revealed 10% to be the optimal plasma concentration. Cultures plated initially in FBS at designated concentrations, then fed with corresponding concentrations of autologous plasma also resulted in enhanced mitogenic responses (Fig 2.2C).

Effect of serum/plasma supplementation on the LPS dose response. Five individual trout were assayed to determine whether autologous plasma resulted in an altered dose response to LPS. The dose of LPS that elicited maximal proliferation did not differ between cultures plated in 10% FBS or cultures plated in 10% autologous plasma, as seen in Figure 2.3. Cultures containing 10% autologous plasma, however, demonstrated a marked increase in their capacity to respond when compared to the same cells cultured in FBS.

Effect of serum/plasma supplementation on the Kknetics of mitogenesis. Kinetic studies were undertaken to determine the number of days required to reach maximal proliferation in response to the optimal concentration of 200 ug/ml of LPS. RBT PBL were cultured for a period of 20 days, under varied supplement conditions (Fig. 2.4). It was observed that day 10 of culture was optimal in the presence of plasma. Cultures utilizing only FBS, showed day 5 of culture was optimal (6690±845 vs 3654±247;1 of 4 experiments) Cells plated either in FBS or autologous plasma, then fed autologous plasma demonstrated an enhanced mitogenic response of up to 60-fold as compared to FBS alone.

Effect of temperature on plasma function. Responses under culture conditions including autologous plasma were found to vary to some degree between individual fish. Initial experiments exploring temperature effects on plasma demonstrated that heat treatment of individual plasma at 56^{o}C for 30 minutes decreased this variability. Plasma samples that initially yielded

good responses were either improved or remained the same when heated to 56°C, and those which demonstrated poor supportive abilities were enhanced by heat treatment. Thus, all subsequent experiments incorporated heat-treated plasma. Heat-treated plasma consistently yielded the highest proliferative response, in some cases increasing the plasma effectiveness up to 62 times that of the same non-treated plasma (Fig. 2.5).

Effect of plasma on other mitogenic responses. Examination of ConA and PHA responsiveness was conducted using previously determined culture conditions above. A representative study of dose responses for ConA and PHA are depicted in Figures 2.6 A and B. The optimal dose of ConA was determined to be 100 ug/ml for all supplement conditions (Fig. 2.6A). Cultures plated in FBS and fed with plasma produced a marked increase in proliferation over those with FBS or autologous plasma alone. PHA elicited very low responses in most fish tested, and responses to different doses of the mitogen varied with the supplement employed. An example of typical results is seen in Figure 2.6B. Cultures supplemented with plasma only demonstrated that 100 ug/ml was optimal, while cells plated initially in FBS and fed plasma resulted in 10 mg/ml as the optimal PHA concentration.

Heterologous plasma effects on the LPS response. Plasma samples taken from several individual fish were examined for their ability to promote mitogenesis as compared to each individual's autologous plasma. Since cells plated in FBS had previously been shown to respond to plasma in a similar manner to cultures exposed to plasma alone, experiments shown in Table 2.1 were performed by plating cells in 10% FBS, then feeding with the various plasma sources at 10%. Results are expressed as stimulation indices (S.I.), and demonstrate the variability between individuals, but the

consistency of enhanced responses in those cultures fed either heterologous or autologous plasma as compared to FBS alone is marked.

DISCUSSION

The results obtained in this study demonstrate the critical nature of the supplements chosen for use in rainbow trout leukocyte culture medium. Our data indicate that the difference between a non-responsive versus a responsive culture may be due solely to the source of supplement chosen for culture. The proliferation potential has been observed to increase up to 60-fold when the cells are cultured in 10% heat-treated trout plasma rather than in the 10% FBS routinely used in cell culture. The degree of enhancement may vary, but in virtually all cases, heat treated plasma has consistently proved to yield higher responses. Figures 1 and 2 demonstrate the vast difference in potential mitogenic responses when the same cells are cultured in medium containing different supplements. All mammalian serum sources tested resulted in relatively low or no mitogen responses. Additionally, Tillit et al. (1988), investigated the role of alternative serum supplements in RBT PBL cultures, utilizing FBS and human serum. It was observed that human serum did not enhance proliferation when compared to FBS supplemented cultures. In our studies the fact that nonresponsiveness in the presence of FBS, HS, and RS was reversed by incorporating trout plasma in the culture medium suggests that all the necessary components for optimal mitogenesis are not present in these serum sources, but may be found in trout plasma.

Etlinger et al. (1976a), in a study of the mitogenic responses of trout lymphocytes, noted that the species source of serum chosen as a medium supplement was capable of markedly affecting mitogenesis. In their study, LPS and purified protein derivative of tuberculin (PPD) stimulation occurred in all organ cultures assayed when medium was supplemented with trout

serum, while stimulation was not observed in medium containing FBS except in the case of PBLs. Stimulation indices (S.I.) for LPS-stimulated PBLs, when cultured in 10% FBS, ranged from 2.0 to 5.2, while the average S.I. for cultures in 20% trout serum was 36. Studies in our laboratory to optimize culture conditions and incubation time focused on the use of LPS as mitogen, because initial results indicated LPS induced the greatest proliferation of all mitogens. LPS dose response assays demonstrated that the optimal mitogen concentration was the same for both plasma and FBS supplementation.

While Etlinger et al. (1976a), found 20% RBTS enhanced the mitogenic response of trout lymphocytes, several investigators (Fryer et al., 1965; Blaxhall, 1985; Collodi and Barnes, 1990) have reported that RBT serum is cytotoxic in fish cell culture at concentrations of 10% and above. Collodi and Barnes (1990), however, have found that 20 ug/ml RBT serum is mitogenic for chinook salmon embryo cells. Blaxhall (1985), in a review of the separation and cultivation of fish lymphocytes, notes that the effect of serum on fish lymphocytes in culture appears to affect trout to a greater degree than carp. Trout serum appears to be inhibitory to cultures, while carp lymphocytes seem to be more tolerant of the homologous serum. This may explain the preference for carp serum in carp lymphocyte culture as opposed to FBS (Caspi et al., 1984; Rosenberg-Wiser and Avtalion, 1982). It appears that investigations into the use of RBT serum for cell culture are inconsistent. Our studies comparing homologous plasma to autologous plasma sources suggest that differences between individuals may be a factor contributing to this variability. Additionally, it appears that serum may not have the same potential as plasma to enhance mitogenicity. Factors in the blood may be affected differently when allowed to clot rather than when

heparinized, perhaps leading to a deprivation of essential components in serum.

Another benefit of using homologous RBT plasma in leukocyte cultures can be seen in Figure 4. Optimal kinetic responses to LPS were observed to occur repeatedly on day 10. This is in contrast to previous RBT mitogen studies which report that maximal responses to all mitogens occur on days 4 or 5 (Etlinger et al., 1976a; Warr and Simon, 1983; Tillit et al., 1988). Tillit et al. (1988), reports that for all concentrations of ConA, proliferation either reached a maximum or declined by day 6. Studies in our laboratory have shown that cultures may be maintained beyond 10 days, though proliferation drops significantly after this time. The ability to maintain cultures in plasma for longer periods of time than those in FBS, with a concomitantly higher capacity for proliferation, may indicate that the rather limited mitogenic response seen with FBS in other laboratories may reflect only the early phase of the mitogenic response. Thus, inclusion of RBT plasma may simply allow for the full expression of the mitogenic response.

Experiments to determine the effects of temperature on the supportive functions of plasma yielded interesting results. Our observations of effects of non heat-treated plasma samples, have suggested that certain individual plasmas may not possess supportive properties. However, if the plasma is heat-treated, supportive properties are then observed. Thus, it appears that certain plasmas may possess suppressive or cytotoxic properties which are heat labile. Therefore, it would be a prudent practice to screen trout plasma sources as well as fetal bovine serum sources for effectiveness, or routinely heat treat plasma samples prior to use, particularly if pooling of several plasma sources is necessary.

Responses to other mitogens demonstrated that plasma mediated enhancement of mitogenic responses was not restricted to LPS. Several experiments in our laboratory revealed that, as opposed to LPS, ConA or PHA demonstrated variable optimal mitogen concentrations for cells cultured in plasma. Etlinger et al. (1976a), also observed that the response to ConA extended over a wider dose range in medium containing RBTS, and required higher doses of ConA for maximal stimulation. Experiments conducted in 20% RBTS supplemented medium required 4-8x as much ConA to produce an optimal response. Results of gel diffusion analyses demonstrated that ConA was capable of precipitating components from either FBS or RBTS, and further, RBTS appeared to contain more of the material capable of interacting with ConA. Etlinger speculated that higher concentrations of ConA should be required to effect maximal stimulation if these serum components were competing with leukocyte receptors. He concluded by postulating that the species source of serum may thus, differentially affect the reactivity of the mitogen.

The essential components for trout lymphocyte mitogenesis that are found in trout plasma but not in FBS, remain to be determined. Overall, our results indicate that much of the inconsistency which has been reported for the use of trout serum can be overcome by the use of plasma. Incorporation of homologous heat treated trout plasma into lymphocyte culture either as a substitute for, or in addition to, FBS may significantly alter previous interpretations of trout lymphocyte mitogenesis, as well as the use of mitogenesis as a tool for assessing lymphocyte heterogeneity.

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Fig. 2.1 **Proliferation of rainbow trout peripheral blood leukocytes (PBL) cultured in medium containing various supplements.** PBLs were cultured either (■) without LPS, or (▨) with LPS at a concentration of 200 µg/ml. PBL cultures were tested in triplicate. A) Cells from a representative fish were plated and fed either 10% horse serum (HS), 10% rabbit serum (RS), or 10% fetal bovine serum (FBS). B) Cells from the same fish were plated in 10% HS, RS, or FBS, then fed 10% autologous trout plasma. C) Cells from an individual fish were plated in FBS and fed 10% trout serum from three different trout serum sources; S1-3. Cells were incubated at 17°C for 10 days. Error bars represent ± 1 standard error.

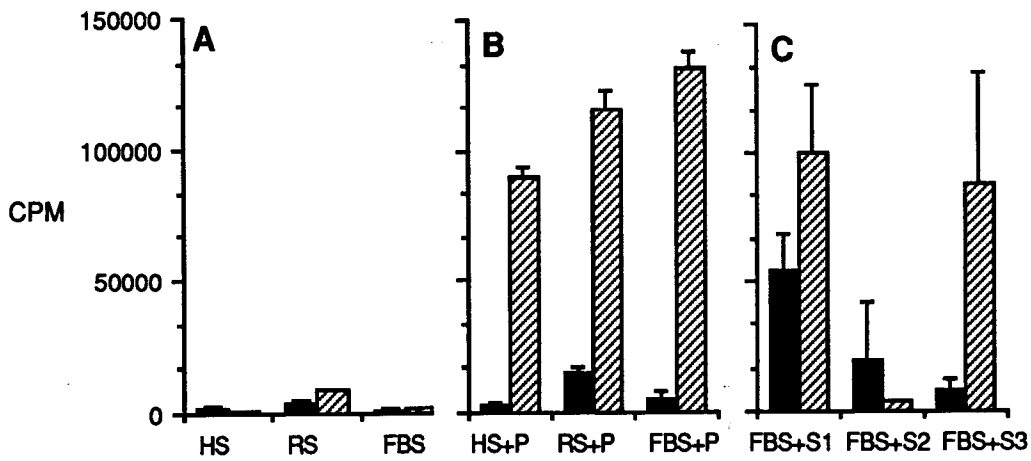

Fig. 2.2 **Proliferation of rainbow trout peripheral blood leukocytes cultured in varying concentrations of supplements.** PBLs were cultured (■) without LPS, or (▨) with LPS at a concentration of 200 µg/ml. PBL cultures were tested in triplicate. Cultures were plated in varying concentrations of : A,C) FBS or B) autologous plasma. Cultures were then fed every third day with the corresponding concentrations of: A) FBS, or B,C) autologous plasma. Concentrations of supplements included 25,10,5, and 1%. Cells were incubated at 17°C for 10 days. This is representative data from one of eight replicate experiments. Error bars represent ± 1 standard error.

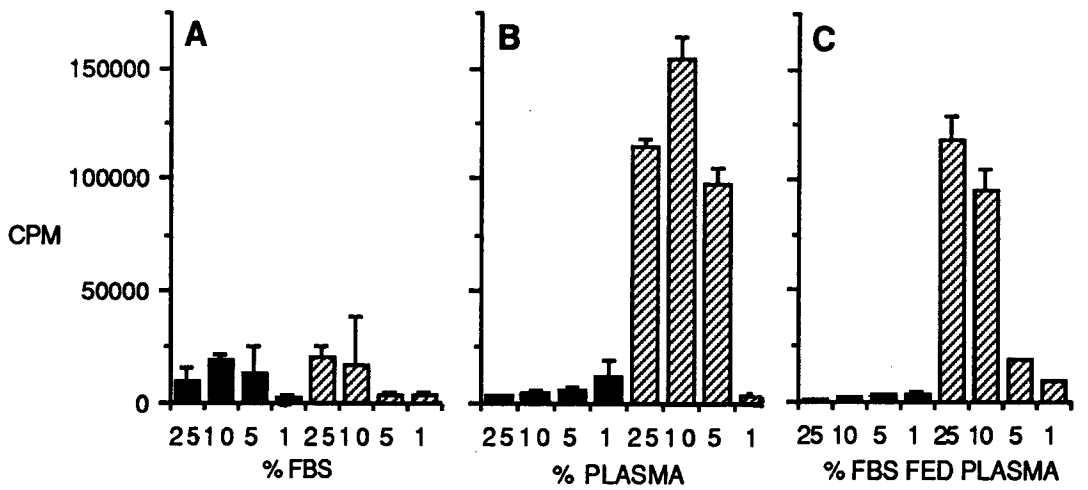

Fig. 2.3 **Responses of leukocytes to various LPS concentrations.** Cells were plated and fed every third day with either 10% (□) rainbow trout plasma, (◆) FBS + rainbow trout plasma, or (■) FBS. Cells were incubated at 17°C for 10 days. This is representative data from one of five replicate experiments. Error bars represent ± 1 standard error.

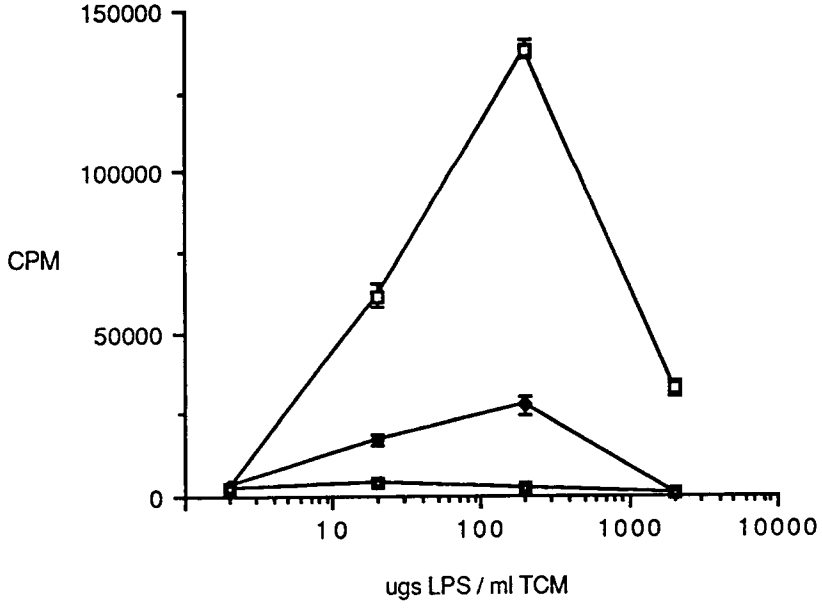

Fig. 2.4 **Kinetic responses of leukocytes to LPS in various supplements.** Cells were plated in and fed every third day with either (□) 10% rainbow trout plasma, (●) 10% FBS + rainbow trout plasma, or (▲) 10% FBS. Cells were harvested every fifth day of culture. Cultures were in triplicate; error bars represent ± 1 standard error. This is representative data from one of six replicate experiments. Cultures without mitogen did not exceed a mean of 2,677 cpm.

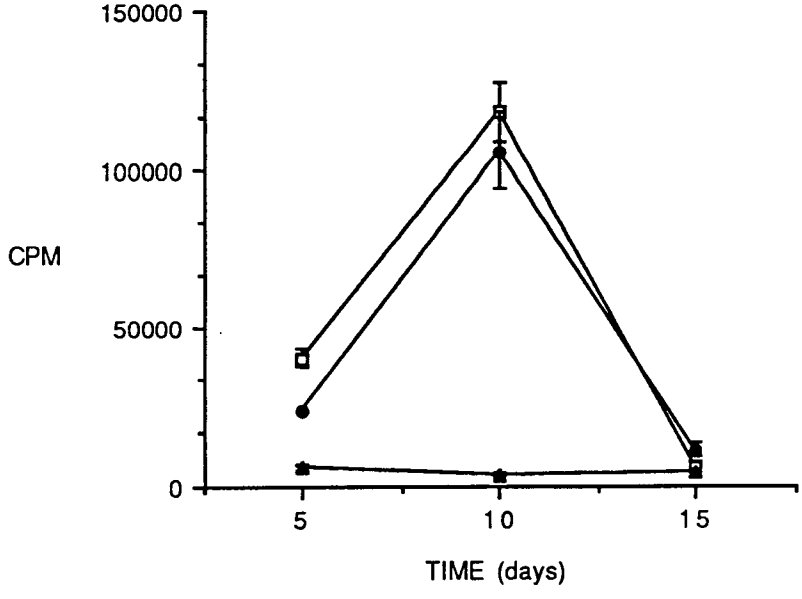

Fig. 2.5 **Kinetic responses of leukocytes to LPS in heat-treated versus non-heat-treated plasma.** Cells were plated in and fed with either (◆) 10% heat-treated rainbow trout plasma, (□) 10% nontreated rainbow trout plasma, or (■) 10% FBS. Cells were harvested every fifth day of culture. Cultures in triplicate; error bars represent + 1 standard error. This is representative data from one of four replicate experiments. Cultures without mitogen did not exceed a mean of 5,694 cpm.

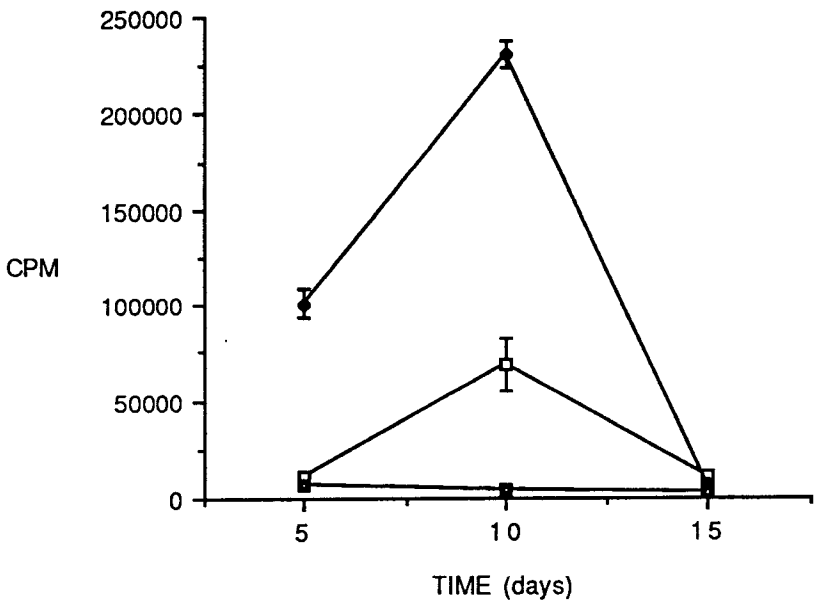

Fig. 2.6 **Dose responses of leukocytes to the mitogens ConA and PHA.** Cells were plated in and fed every third day with either (□) 10% rainbow trout plasma, (◆) 10% FBS plated and plasma fed, or (■)10% FBS. Cultures were incubated at 17°C for 10 days. Cultures were tested in triplicate; error bars represent ± 1 standard error. This is representative data from two of six replicate experiments.

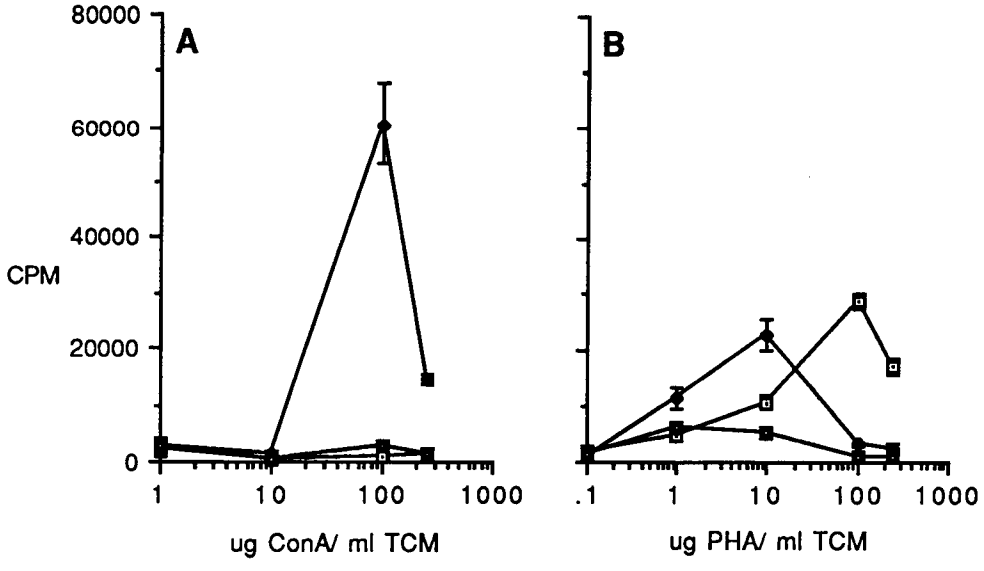

Table 2.1 **Comparison of stimulation indices obtained from various sources of rainbow trout leukocytes at an optimal concentration of LPS.** [a] Values obtained from six individual experiments. Cultures in triplicate and harvested on day 10. [b] Assays conducted by plating cells in FBS and feeding with FBS. [c] Assays conducted by plating cells in FBS and feeding with autologous heat inactivated plasma. [d] Assays conducted by plating cells in FBS and feeding with homologous heat inactivated plasma. [e] Results indicated as stimulation indices (cpm with LPS/ cpm without LPS). [f] Not determined.

	Trout Plasma Source				
TEST #[a]	FBS[b]	AP[c]	HP1[d]	HP2	HP3
1	10.0[e]	34.0	54.0	65.0	ND[f]
2	0.54	27.0	10.0	36.0	12.0
3	0.98	12.0	27.0	28.0	11.0
4	1.50	11.0	18.0	21.0	19.0
5	1.10	28.0	29.0	12.0	ND
6	2.30	99.0	120.0	100.0	20.0

CHAPTER 3

An Improved Salmonid Lymphocyte Culture Medium For In Vitro Antibody Production and Mitogenesis

Jenefer DeKoning and Stephen Kaattari

SUMMARY

This study describes an improved culture medium utilizing salmonid plasma sources as an alternate serum supplement to fetal bovine serum (FBS). Salmonid leukocyte antibody and mitogen responses were examined in cultures supplemented with either salmonid plasma or FBS. Salmonid plasma sources repeatedly enhanced the mitogen specific proliferation of the leukocyte cultures above that seen with FBS. Additionally, antibody responses of these cultures were enhanced when plasma was employed, demonstrating factor(s) present in plasma were necessary for differentiation as well as proliferation. This phenomena was observed for both peripheral blood and anterior kidney leukocytes. A comparison of nonspecific stimulation of cultures in the absence of mitogen revealed FBS, alone, frequently elicited significant proliferation, while plasma supplemented cultures did not. Utilization of plasma as an alternate serum supplement has broad applications as enhancement of the mitogenic response was observed for three distinct salmonid species. In addition plasma sources were effective across species, suggesting plasma supplementation may be adapted to many fish systems.

INTRODUCTION

Fetal bovine serum (FBS) has been routinely used in fish cell culture as the preferred serum supplement (Nicholson, 1989), since the sera and media used in fish cell culture have been adapted from mammalian cell culture procedures with little or no modification (Wolf and Mann, 1980). Recently it has been shown that use of homologous plasma in rainbow trout peripheral blood leukocyte culture results in an enhanced mitogenic response when compared to FBS (DeKoning and Kaattari, 1991). Studies with warm water fish species such as catfish and carp have found alternatives to FBS; commonly incorporating homologous serum (Faulmann *et al.*, 1983; Cuchens and Clem, 1977; Rosenberg-Wiser and Avtalion, 1982; Caspi *et al.*, 1984), or serum free medium (Luft et al., 1991). Studies with the cold water salmonid species though, have continued to utilize FBS as the primary serum supplement (Warr and Simon, 1983; Blaxhall, 1985; Tillit *et al.*, 1988).

This FBS utilization may also be due to the inhibitory effect trout serum has been reported to exhibit on leukocyte cultures (Blaxhall, 1985). However, earlier studies (Etlinger *et al.*, 1976b; Tillit *et al.*, 1988) on the effect of alternative serum supplements to FBS on trout mitogenesis, have shown that FBS yields varying results from spontaneous mitogenic activity to an inability to support mitogen-induced reactivity. Etlinger *et al.* (1976a), demonstrated that trout serum was often superior to FBS in supporting mitogenic responses. Further, it has been suggested in studies designed to assess lymphocyte functional heterogeneity, that an apparent absence of responsive cells actually may be due to suboptimal culture conditions (Caspi *et al.*, 1984; Rosenberg-Wiser and Avtalion, 1982; Tillit, *et al.*, 1988).

Evidence presented recently from our laboratory supports this hypothesis (DeKoning and Kaattari, 1991). Our studies reveal that FBS does not allow for maximal mitogenic stimulation of lymphocytes. The controversial results to date appear to be rectified by utilizing trout plasma instead of FBS or trout serum.

In this report we have extended our studies in trout to other salmonid species to examine the general applicability of utilizing homologous plasma as the primary serum source for salmonid leukocyte culture. In addition we have demonstrated the usefulness of plasma in assessing immunocompetency in salmonids, and potential for use in further functional studies.

MATERIALS AND METHODS

Animals. Coho salmon (*Oncorhynchus kisutch*), spring chinook salmon (*Oncorhynchus tshawytscha*), and rainbow trout (*Oncorhynchus mykiss*) were maintained at the Smith Farm Experimental Hatchery in Corvallis, OR. This facility receives fish pathogen-free water at a constant temperature of 12°C. Fish weight ranged from 300 - 800 grams. Fish were fed Oregon Moist Pellet (OMP) commercial salmon food daily.

Culture medium. Lymphocytes were cultured either in tissue culture medium (TCM) consisting of RPMI-1640 with sodium bicarbonate supplemented with 10% defined Fetal Bovine Serum, lot #1111889 (Hyclone, Logan, UT), or 2% salmonid plasma (unless otherwise noted), and 0.05 mg/ml gentamycin sulfate (Whittaker Bioproducts Inc.). Plasma was obtained as described below, then diluted to 10% in RPMI (containing no anticoagulant). The diluted plasma was subsequently heat treated for 30 minutes at 56°C. Final plasma concentrations were obtained by further dilutions in RPMI.

Cell preparation and tissue culture. Anterior kidney tissue, peripheral blood leukocytes and plasma were obtained from fish sacrificed by anesthetic overdose in benzocaine (ethyl p-aminobenzoate, Sigma) (Kaattari and Irwin, 1985). Caudal bleeds were taken in 10 ml sodium heparin Vacutainer tubes (Becton Dickinson and Co., Rutherford, NJ.). The whole blood was then centrifuged at 17°C in 15 ml conical polystyrene tubes (Corning Glass Works, Corning, NY) for 10 minutes at 500 x g to pellet the blood cells. The plasma was removed and saved, and the blood cells were resuspended in RPMI-1640 (Gibco Laboratories, Grand Island, NY) to approximately 5 times their original volume, then layered on an equivalent

volume of Histopaque-Ficoll 1077 (Sigma). The tubes were centrifuged at 17°C for 45 minutes at 500 x g. The interface layer of cells between the RPMI and Histopaque was recovered and washed twice in RPMI at 500 x g for 10 minutes at 17°C. Cells were then resuspended in RPMI and viability determined by trypan blue dye exclusion.

Anterior kidney tissue was harvested by dissection and immersed in RPMI. A single cell suspension was prepared by grinding tissue through a Cellector tissue sieve (VWR Scientific, Seattle, WA). Cells were washed twice in RPMI at 500 x g for 10 minutes at 17°C. Cells were then resuspended in RPMI and viability determined by trypan blue dye exclusion.

After enumeration, cells were resuspended to a concentration of 1 x 10^7 cells/ml in either TCM or RPMI with 2% plasma. Aliquots of 100 µl were cultured in individual wells of a 96 well flat bottom tissue culture plate (Corning Glass Works, Corning, NY). LPS, or TNP-LPS in appropriate concentrations were then added in 10 µl volumes. Cells were incubated in an incubator culture chamber (Model 624, C.B.S. Scientific Co., Del Mar, CA) at 17°C. in a blood-gas environment containing 10% CO_2, 10% O_2, 80% N_2.

Mitogen and antigen preparation. Stock solutions of *E. coli* 055.B5 lipopolysaccharide (LPS) (Sigma Chemical Company, St. Louis, MO) were diluted in RPMI to a concentration of 10 mg/ml and pasteurized by incubation in a 70°C water bath for 1 hour.

Trinitrophenylated-lipopolysaccharide (TNP-LPS) was prepared by the method of Jacobs and Morrison (1975). TNP-LPS was pasteurized for 45 minutes in a 70°C water bath and stored at 4°C.

Mitogen assay. Twenty hours prior to harvest, 10 µl (1 µCi) of 3H-thymidine, specific activity of 6.7 Ci/mmole, (ICN Biomedicals, Inc., Irvine,

CA) diluted in RPMI was added to each well. Cells were harvested as previously described by Kaattari and Yui (1987).

Plaque forming cell assay. On the day of harvest, 96 well culture plates were centrifuged for 5 minutes at 500 x g. The culture supernatants were removed and cells were resuspended in 200 µl RPMI per well. Cells secreting anti-TNP antibody were then detected by methods previously described by Kaattari and Yui (1987).

RESULTS

Plasma dose response analysis. Coho salmon peripheral blood leukocytes were cultured in various concentrations of autologous plasma, or 10% FBS, in the presence of an optimal concentration of lipopolysaccharide (LPS). Figure 3.1 shows four individual dose responses to autologous plasma. In all cases, an optimal concentration of plasma either enhanced or maintained the mitogen response as compared to FBS. Four individual responses were chosen to depict the variability of the optimal concentration, yet the enhancement is consistently observed between individuals. A plasma concentration of 2% was chosen as optimal after fifteen repetitions of this assay.

Kinetic analysis. Kinetic studies of rainbow trout peripheral blood leukocytes previously revealed an altered kinetic response for cells cultured in plasma as compared to FBS. Figure 3.2 demonstrates that the kinetics of the mitogen response to LPS of coho salmon leukocytes is also different for cells cultured in plasma versus FBS. Plasma cultured leukocytes from a representative individual show that a peak response occurs at approximately day 6, while the same cells when cultured in FBS show maximal proliferation at or before day 4 of culture.

Correlation of enhanced mitogenesis and antibody production. Autologous plasma was examined for its capacity to enhance *in vitro* antibody production. Figures 3.3A demonstrates the peripheral blood lymphocyte response to the mitogen LPS in the presence of plasma and FBS. Aliquots of these same cells were also simultaneously tested for their ability to produce an antibody response to TNP-LPS, such that a direct comparison of the plasma effects on both functions could be made. It was

demonstrated that plasma-induced enhancement was not limited to the proliferative phase (mitogenesis) but also stimulated plasma cell differentiation.

Figure 3.3B demonstrated the same enhancement for anterior kidney leukocytes. Anterior kidney cells were cultured in a similar manner and assayed for either their mitogen response to LPS or their plaque forming cell response to TNP-LPS. Antibody production and corresponding mitogen response were again enhanced for anterior kidney leukocyte cultures supplemented by plasma. Additionally, it should be noted that neither plasma or FBS alone induced polyclonal stimulation of antibody production (-TNP-LPS, Fig. 3.3A,B).

Nonspecific stimulation by FBS. Cultures supplemented with FBS alone (no mitogen) exhibited consistently greater proliferation than those cultures supplemented with plasma. Figure 3.4A depicts the stimulation indices (SI) for six individual responses to LPS for leukocytes cultured either in plasma or FBS. The background counts per minute (cpms) for six cultures are shown in figure 3.4B. FBS supplemented cultures in the absence of mitogen exhibit higher CPMs, leading to decreased SIs, when compared to plasma supplemented cultures. Thus FBS frequently induces nonspecific stimulation which can, in part, obscure the magnitude of the mitogen-induced response.

Autologous versus pooled plasma effectiveness. Attempts at optimization of plasma concentration has yielded variable responses depending on the individual serum source. Therefore, a stock plasma source consisting of pooled plasma from four individuals was assayed for its effectiveness in the mitogen response. Figure 3.5 depicts a comparison of the mitogen response of leukocytes cultured in one of the autologous

plasmas versus pooled plasma. This figure demonstrates the effectiveness of a pooled plasma source and suggests this may be a viable option for eliminating autologous plasma concentration variability between individuals.

Heterologous plasma from three salmonid species. Plasma samples were taken from rainbow trout, coho and chinook salmon and assayed for the ability of each to enhance proliferation of leukocytes from these three species. Figure 3.6 demonstrates the responses of 1 rainbow trout, 3 coho, and 3 chinook leukocyte cultures to these various plasma species sources. Though variability exists in the optimal plasma concentration and in effectiveness between species, all plasma sources enhanced the degree of mitogenesis as compared to FBS. This cross-species supportive capability among these species suggests a general salmonid plasma source can be used as an alternative to FBS supplementation and improve salmonid leukocyte culture conditions.

DISCUSSION

The results presented here demonstrate that salmonid leukocyte mitogenesis and antibody production is enhanced in cultures supplemented with an optimal concentration of plasma. Our data strongly indicate that the more routinely used serum supplement, FBS, results in a suboptimal culture medium. Additionally, FBS has often been observed to be unsupportive of mitogenic responses as evidenced by non-responsive cultures which, when supplemented with plasma, become responsive (DeKoning and Kaattari, 1991).

Investigations into alternative serum supplements for salmonid leukocyte cultures have been sparse. The few studies to date have focused on rainbow trout leukocytes (Etlinger et al., 1976a; Etlinger et al., 1976b; Tillit et al., 1988; Warr & Simon, 1980). Previous results in our laboratory have established that incorporation of autologous or homologous plasma in rainbow trout peripheral blood leukocyte cultures markedly increased the proliferative potential as compared to FBS. The present study was undertaken to determine the applicability of this culture method to other salmonid species, and to the *in vitro* production of antibodies.

In determining the optimal plasma concentration for use in culture, multiple autologous plasma dose response analyses were performed. Variability was observed to exist between individuals and in some cases, high concentrations of plasma may actually inhibit proliferation of cultures (Fig. 3.1). However, at an optimal concentration, plasma routinely enhances mitogenesis when compared to FBS. Plasma supplementation not only increased the overall proliferative potential of salmon leukocytes, but also increased the number of days required to achieve a peak response (Fig.

3.2). Results shown are representative data from several replicate experiments. These results were in agreement with previous studies on rainbow trout leukocyte cultures (DeKoning and Kaattari, 1991).

In order to assess the general applicability of plasma supplementation, the effectiveness of plasma in enhancing *in vitro* peripheral blood leukocyte antibody production was assessed. Plasma clearly enhances B cell differentiation through to the generation of plasma cells (Fig 3.3). All necessary factors present in plasma do not appear to be present or active in FBS. Alternatively, essential factors may be present in FBS, but may be accompanied by inhibitory factors or concentrations of factors. A continuation of this analysis was also performed using anterior kidney cells (Fig. 3.3B). Results demonstrated plasma enhancement is not limited to peripheral blood cells.

Previously, others have demonstrated the variable and sometimes deleterious effects of FBS on leukocyte culture (Etlinger et al., 1976a; Etlinger et al., 1976b; Tillit et al., 1988). In our studies we have observed FBS effects ranging from stimulation in the absence of mitogens (Fig. 3.4), to inhibition of both mitogen and antibody responses. A portion of the enhanced stimulation indices in plasma supplemented cultures is due to the fact that plasma reduces nonspecific stimulation. Background cpms for plasma supplemented cultures are consistently lower than FBS supplemented cultures. This, in turn, leads to increased stimulation indices and a clearer elucidation of the specific response. However, regardless of background cpms, total cpms for plasma supplemented cultures are still at least 3 - 4 fold higher than those seen with FBS.

The discovery that coho salmonid leukocytes responded similarly with plasma as do rainbow trout leukocytes, suggested that these two plasma

sources shared a common property or factor(s). Therefore, we sought to determine if each species' plasma would show a similar enhancement with other species' leukocytes. Plasma samples from three species were compared for their effectiveness on each of these species' peripheral blood leukocytes. Though variability did exist as to the optimal concentration for each plasma source, all plasma sources, at their respective optimal concentration, enhanced the mitogenic response in all other cultures over that seen with FBS (Fig. 3.6).

These data support the idea that FBS does not provide the essential conditions for salmonid leukocyte culture. Alternatively, it has been shown that benefits of plasma supplementation are not limited to one cell source or one species. Plasma supplementation is an improved culture method that may be potentially adapted to other fish leukocyte culture systems.

ACKNOWLEDGEMENTS

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Fig. 3.1 **Proliferation of coho salmon peripheral blood leukocytes to LPS cultured in varying concentrations of autologous plasma or 10% FBS.** Concentrations of plasma included 5%, 2%,0.5% and 0.05%. LPS was used at an optimal concentration of 200 μg/ml. PBL cultures were tested in triplicate, cells were incubated for 6 days at 17°C. Error bars represent one standard deviation. Cultures in the absence of mitogen did not exceed a mean of 2,238 ± 320 cpm.

Fig. 3.1

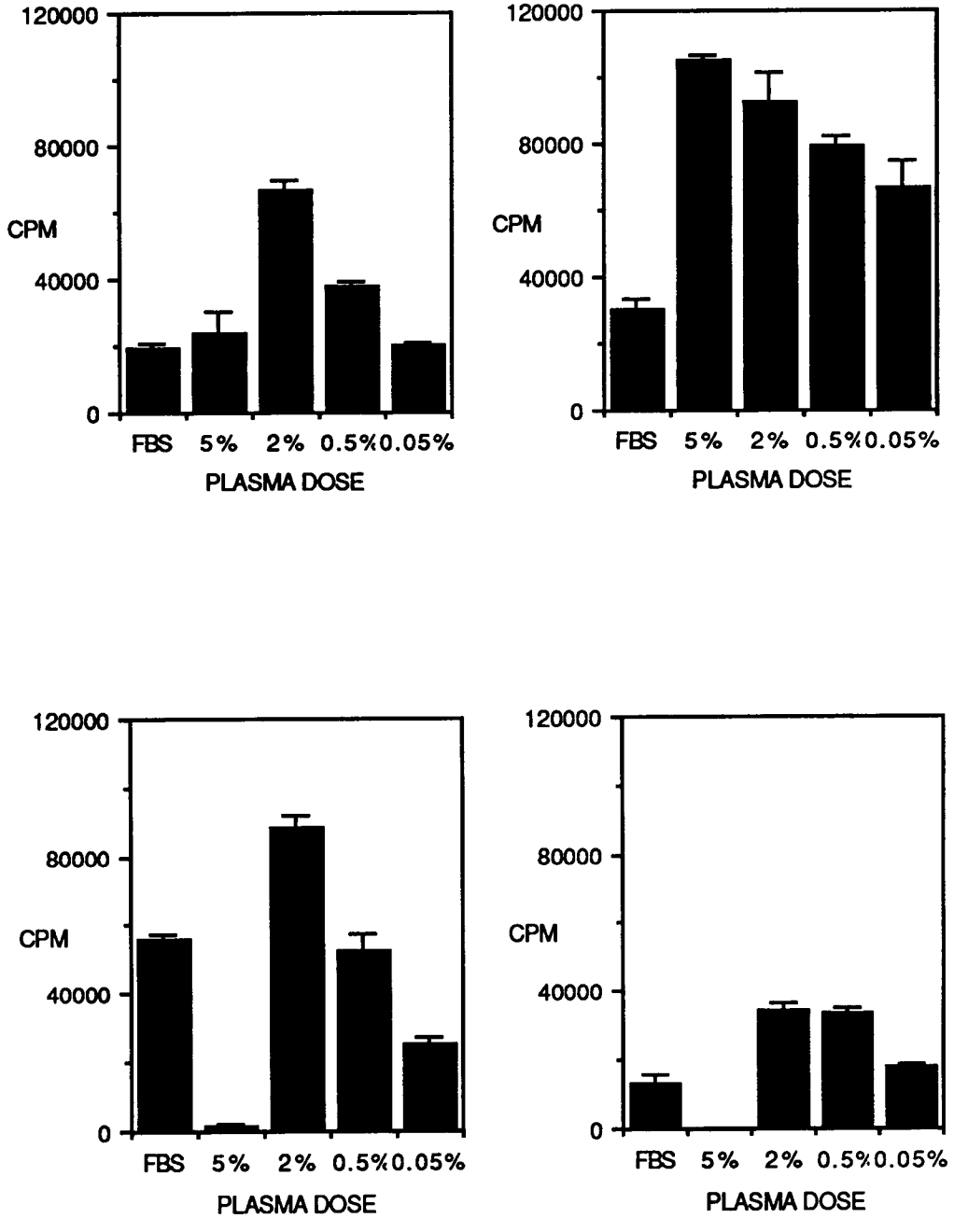
120000
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Fig. 3.2 **The kinetic response of peripheral blood leukocytes to LPS when supplemented either with plasma or FBS.** Cells were plated in either (◆) 2% plasma or (□) 10% FBS. Cells were harvested on days 4,6,8, and 10 of culture. Cultures were in triplicate, and incubated at 17°C. Error bars represent one standard deviation. Cultures in the absence of mitogen did not exceed a mean of 2,246 ± 749 cpm.

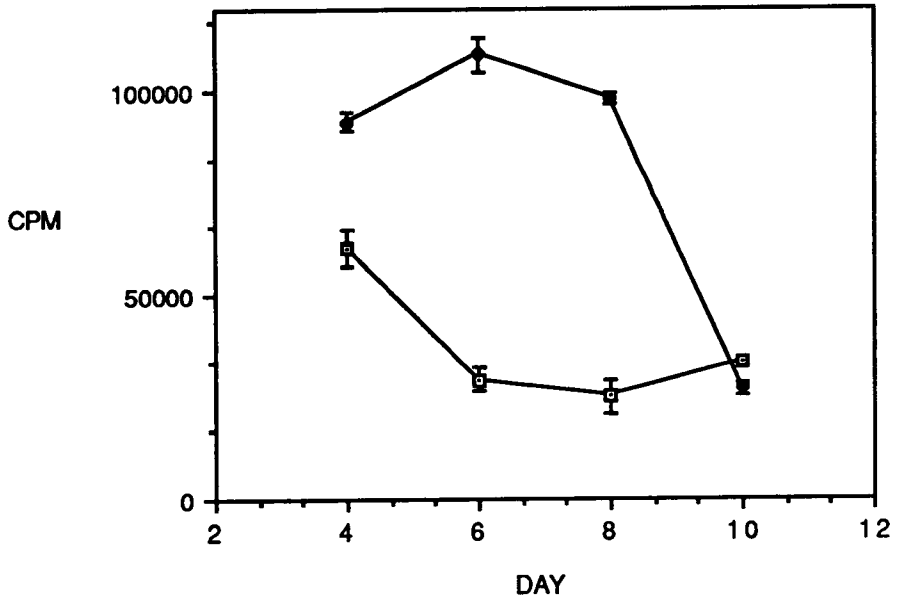
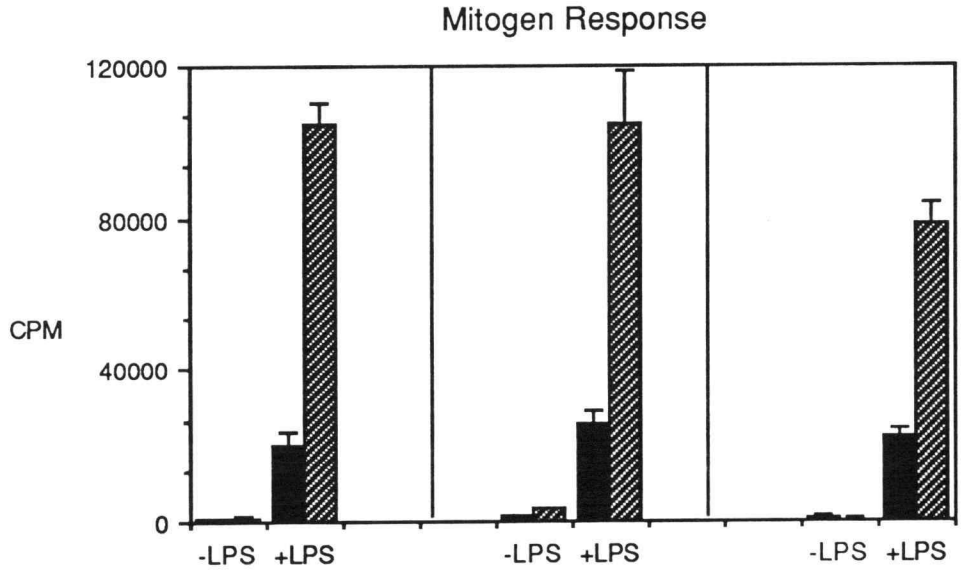
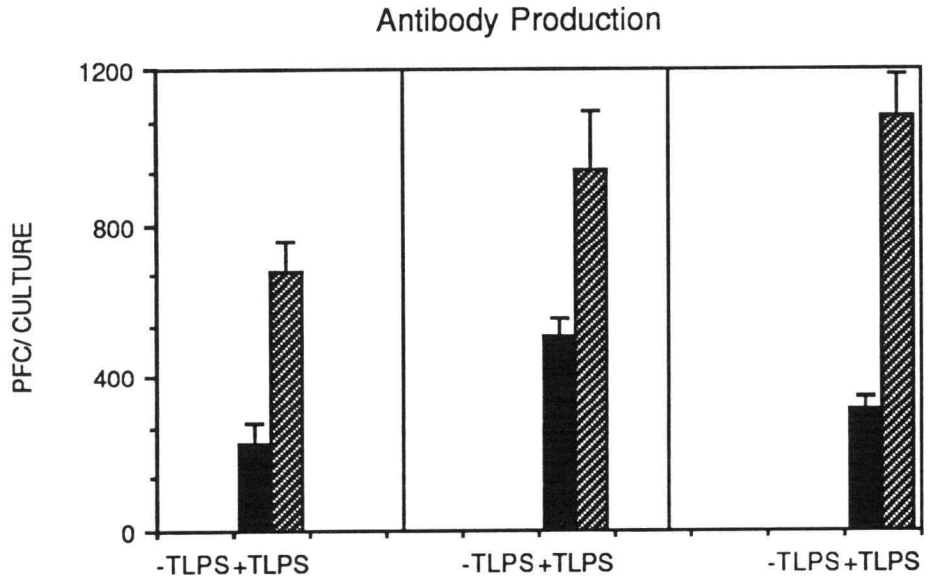


Fig. 3.3 **The enhancement of mitogenic responses and plaque forming cell responses for coho salmonid leukocytes.** Cultures of the leukocytes from three fish were supplemented either with (▨) 2% plasma, or (■) 10% FBS. Peripheral blood leukocytes (A) were cultured in the presence of LPS for the mitogen response (top panels), or TNP-LPS for the PFC response (bottom panels). Anterior kidney cells (B) were cultured in the presence of LPS for the mitogen response, or TNP-LPS for the PFC response as above. Mitogen cultures were in triplicates, and were harvested on day 6 of culture. PFC cultures were in quadruplicates, and were harvested on day 8 of culture. Error bars represent 1 standard deviation. Cultures in the absence of mitogen/antigen did not exceed a mean of 4,718 + 1652 cpm, or 10 + 7 PFC / cultures.

Fig. 3.3A


Mitogen Response
120000
80000
40000
0
CPM
-LPS +LPS
-LPS +LPS
-LPS +LPS


Antibody Production
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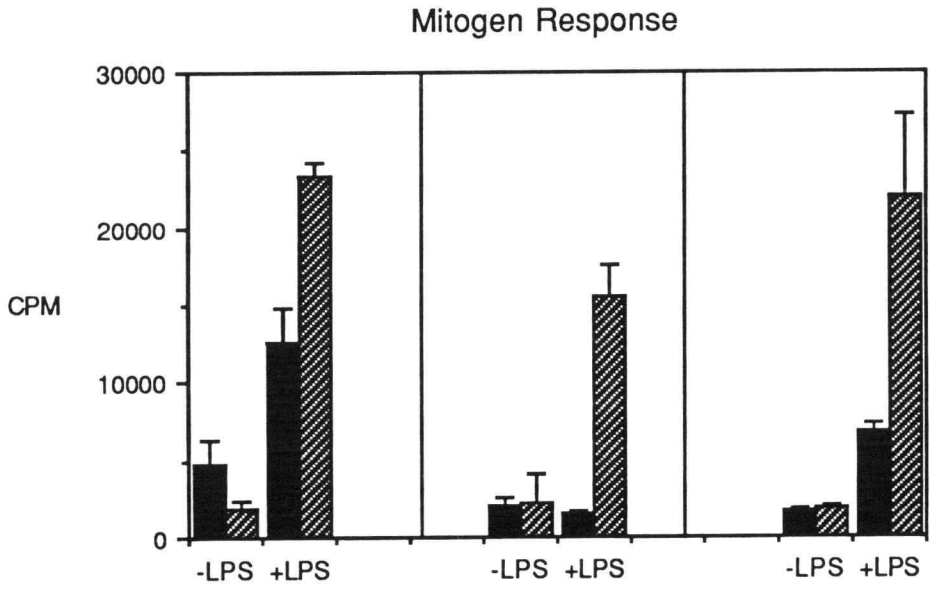
800
400
0
PFC/CULTURE
-TLPS+TLPS
-TLPS+TLPS
-TLPS+TLPS

Fig. 3.3 B


Mitogen Response
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20000
10000
0
CPM
-LPS +LPS
-LPS +LPS
-LPS +LPS

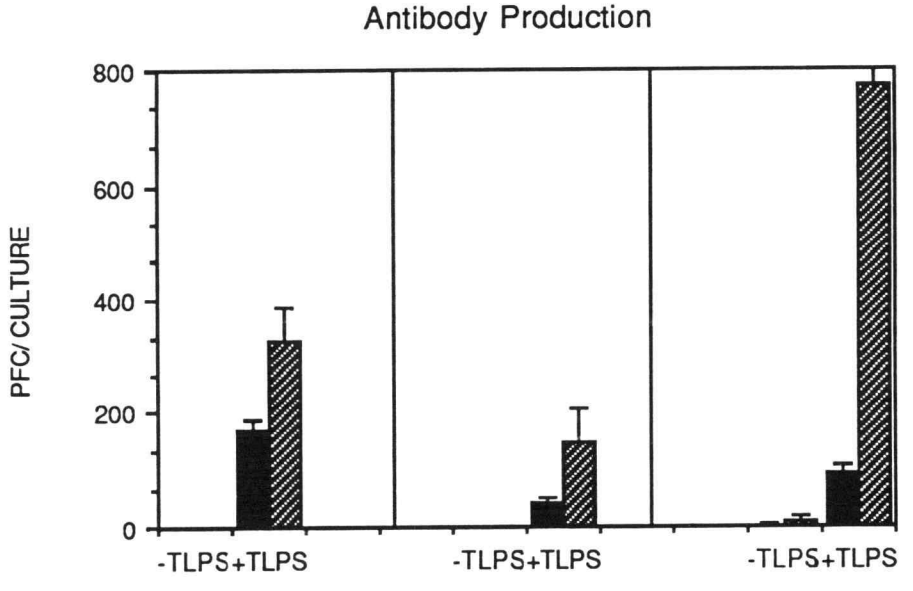
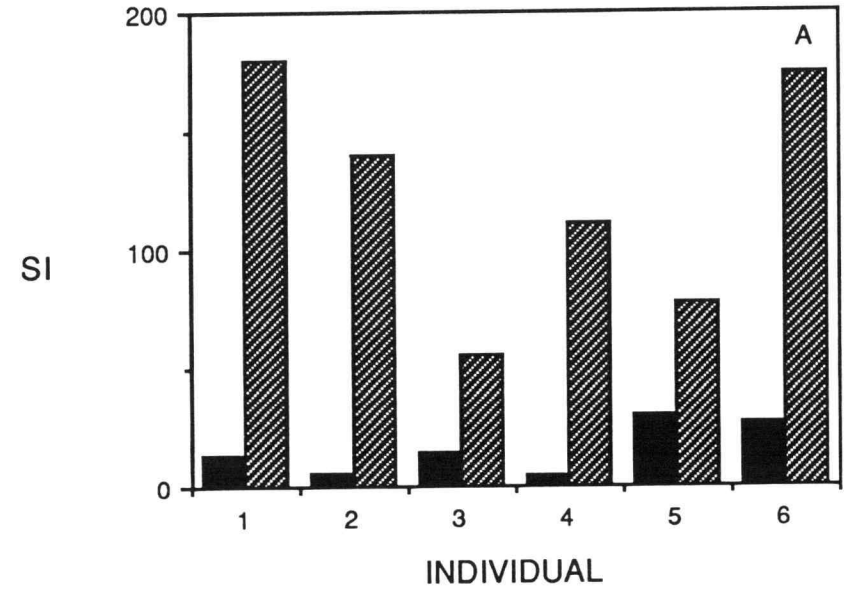

Antibody Production
800
600
400
200
0
PFC/CULTURE
-TLPS+TLPS
-TLPS+TLPS
-TLPS+TLPS

Fig. 3.4 **Comparison of stimulation indices and background cpms for leukocytes supplemented with either plasma or FBS.** (A) Peripheral blood leukocytes from six individuals were cultured in the presence of LPS, and supplemented with either (▨) 2% plasma or (■) 10% FBS. The results are expressed as stimulation indices (cpm with LPS / cpm without LPS). Cultures in the absence of mitogen did not exceed a mean of 3,534 ± 1,104 cpm. (B) Peripheral blood leukocytes from six different individuals were cultured in the absence of LPS and results expressed as cpms. Error bars represent one standard deviation.

Fig. 3.4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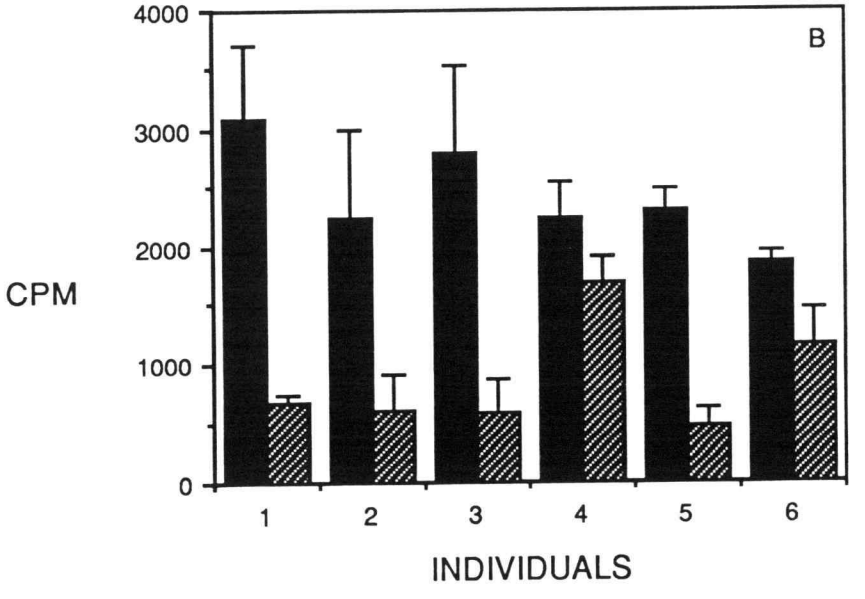

4000
3000
2000
1000
0
CPM
B
1
2
3
4
5
6
INDIVIDUALS

Fig. 3.5 **Proliferation of coho salmonid peripheral blood leukocytes cultured in LPS, supplemented in varying concentrations of autologous or pooled plasma, or 10% FBS.** Concentrations of autologous or pooled plasma include (▨) 5%, (▩) 2%, (▧) 0.5%, and (□) 0.05%. (■) FBS was used at a concentration of 10%. Cultures were in triplicate and error bars represent one standard deviation. Cultures without mitogen did not exceed a mean of 1,367 ± 366 cpm.

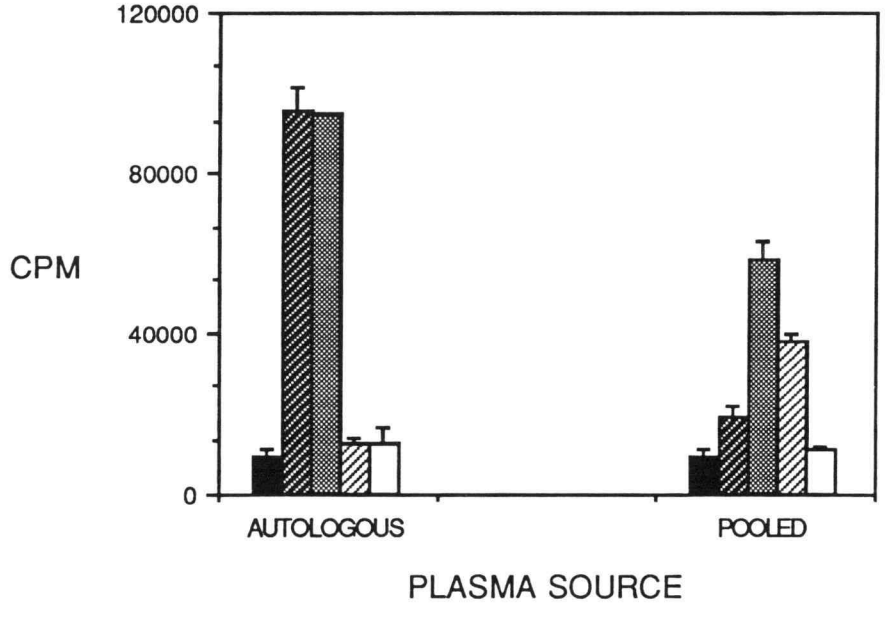
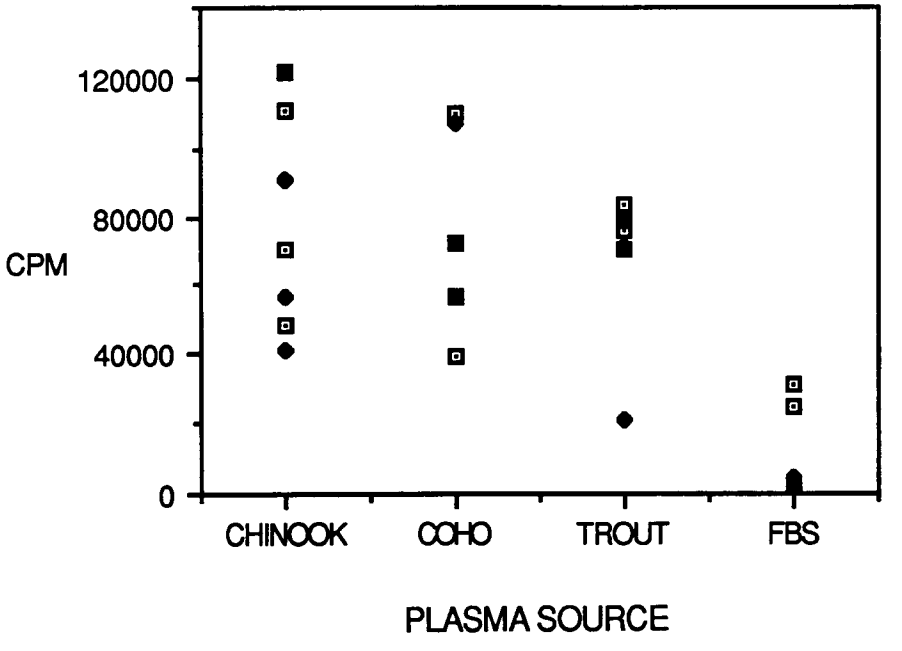


Fig. 3.6 **The mitogen response of coho, chinook, and rainbow trout peripheral blood leukocytes supplemented with either coho, chinook, or rainbow trout plasma, or FBS.** Cells were cultured in the presence of LPS. Species sources of leukocytes represented by (□) coho, (◆) chinook, or (■) rainbow trout. Data from three coho, three chinook and one rainbow trout are represented. Cultures were in triplicate, and error bars represent one standard deviation. Cultures without mitogen did not exceed a mean of 18,066 ± 1,848 cpm for rainbow trout, 2,225 ± 490 for chinook, and 4,397 ± 190 for coho.



Chapter 4

A Natural Regulatory Cell Population Located in the Anterior Kidney of Coho Salmon (*Oncorhynchus kisutch*)

Jenefer DeKoning, Hank Ortega, and Stephen Kaattari

Coauthor contribution: J.D., primary investigator; H.O., technical assistant; S.K., research director

SUMMARY

In this report we present evidence for the existence of a natural regulatory cell population located in the anterior kidney. The addition of anterior kidney cells (AKC) to either autologous peripheral blood or spleen cell cultures results in suppression of the proliferative response to the mitogen, lipopolysaccharide. The degree of suppressor activity exhibited by AKC appears to be correlated with the ability of the anterior kidney to respond to mitogenic stimulus. A decrease in the mitogen response of the anterior kidney is significantly correlated with an increase in the suppressor activity exerted upon peripheral blood lymphocytes. Additionally, AKC effects upon peripheral blood and spleen plaque forming cell responses were examined. Similar to the mitogen response results, AKC addition to spleen cultures resulted in a significant suppression of the antibody response. In contrast, addition of AKC to peripheral blood lymphocyte plaque forming cell cultures resulted either in no effect or enhancement of the antibody response in the majority of experiments. Effects of low dose irradiation (400-800 rads) on the mitogen response of the peripheral blood, spleen and anterior kidney were also examined. This analysis revealed a radiosensitive suppressor population in the peripheral blood and spleen, while no such population appears to exist within the anterior kidney. We postulate that the suppressor population found in the anterior kidney is radioresistant, and may possess an important immunoregulatory function.

INTRODUCTION

In the teleost, the sites of lymphohematopoietic activity include the anterior kidney, thymus and spleen (Zapata, 1979; Zapata and Cooper, 1990). It is currently believed that the anterior kidney is the primary hematopoietic organ in the teleost and, as such, has been compared to mammalian bone marrow in function. Every line of hematopoietic differentiation has been observed in the kidney including pluripotent stem cells, as well as immature and mature red and white blood cells (Smith et al., 1970; Zapata, 1979; Al-Adhami and Kunz, 1976). However, all cellular components of the teleost immune system have not been clearly differentiated. As the primary hematopoietic organ, possessing cells in all stages of maturation, the anterior kidney would be suspected to be the target of complex and exquisite regulatory mechanisms. This has been observed in the mammalian counterpart to the anterior kidney, the bone marrow (Kincade et al., 1989). The existence of cells governing such immunoregulation, or their identity has yet to be addressed in teleosts.

In mammals, the bone marrow, considered to be the counterpart to the fish anterior kidney, is a complex organ that is the major source of all hematopoietic cells (Owen et al., 1977; Corvese et al., 1980; Levitt and Cooper, 1980). Several reports have documented the presence of natural regulatory cells in the bone marrow capable of regulating various immunologic reactions (Duwe and Singhal, 1979; Dorshkind et al., 1980; Mortari et al., 1986). There are several subpopulations of cells comprising the natural immune system which are capable of antigen-nonspecific, MHC-unrestricted cell killing or suppression (Maier et al., 1986; Maier et al., 1989). All are considered members of the large granular lymphocyte (LGL) family.

This family includes natural killer (NK) cells, natural cytotoxic (NC) cells and natural suppressor (NS) cells. These cell types are diverse, making it difficult to precisely differentiate their functional and phenotypic characteristics, but the major function identified for these cells is their suppressive capacity.

Previous studies examining the components of the natural immune system in salmonids (Moody et al., 1985), carp (Hinuma et al., 1980), and catfish (Graves et al., 1984; Evans et al., 1984a,b; Evans et al., 1987) have discovered the presence of natural cytotoxic cells (NCC) capable of spontaneously lysing various mammalian and teleost cell lines. These cytotoxic cells have been shown to be antigen-nonspecific, require cell-cell contact for target cell lysis, and are similar morphologically to mammalian natural killer cells (NK). In fish, all components comprising the natural immune system have not yet been so well defined. To date, the few studies in this broad area have focused on the above mentioned NCC cells, which have only been characterized as to their cytotoxic activity, and have not been examined for suppressor function. In this report, we present evidence of a cell population within the anterior kidney which exhibits suppressor activity in autologous mitogen responses. This is the first evidence of a natural immunoregulatory cell reminiscent of the natural suppressor (NS) cell seen in mammals.

MATERIALS AND METHODS

Animals. Coho salmon (*Oncorhynchus kisutch*) were maintained at the Smith Farm Experimental Hatchery in Corvallis, OR. This facility receives fish pathogen-free water at a constant temperature of 12°C. Fish weight ranged from 300 - 800 grams. Fish were fed Oregon Moist Pellet (OMP) commercial salmon food daily.

Mitogens and antigens. Stock solutions of *E. coli* 055.B5 lipopolysaccharide (LPS) (Sigma Chemical Company, St. Louis, MO) were diluted in RPMI-1640 to a concentration of 10 mg/ml and pasteurized by incubation in a 70°C water bath for 1 hour. Trinitrophenylated-lipopolysaccharide (TNP-LPS) was prepared by the method of Jacobs and Morrison (1975). TNP-LPS was pasteurized for 45 minutes in a 70°C water bath and stored at 4°C. Free hapten, TNP-lysine (ε-TNP-L-Lysine Monohydrochloride, ICN Biochemicals, Cleveland, OH), was prepared at a stock concentration of 10^{-3} M in RPMI-1640 and filter sterilized.

Cell preparation and tissue culture. Anterior kidney tissue, peripheral blood leukocytes and plasma were obtained from fish sacrificed by anesthetic overdose in benzocaine (ethyl p-aminobenzoate, Sigma) (Kaattari and Irwin, 1985). Caudal bleeds were taken in 10 ml sodium heparin Vacutainer tubes (Becton Dickinson and Co., Rutherford, NJ.). The whole blood was centrifuged at 17°C in 15 ml conical polystyrene tubes (Corning Glass Works, Corning, NY) for 10 minutes at 500 x g to pellet the blood cells. The plasma was removed and saved. The blood cells were resuspended in RPMI-1640 (Gibco Laboratories, Grand Island, NY) to approximately 5 times their original volume, then layered on an equivalent volume of Histopaque-Ficoll 1077 (Sigma). The tubes were centrifuged at

17°C for 45 minutes at 500 x g. The interface layer of cells between the RPMI and Histopaque was recovered and washed twice in RPMI at 500 x g for 10 minutes at 17°C. Cells were then resuspended in RPMI and viability determined by trypan blue dye exclusion.

Anterior kidney tissue was harvested by dissection and immersed in RPMI. A single cell suspension was prepared by grinding tissue through a Cellector tissue sieve (VWR Scientific, Seattle, WA). Cells were washed twice in RPMI at 500 x g for 10 minutes at 17°C. Cells were then resuspended in RPMI and viability determined by trypan blue dye exclusion.

After enumeration, cells were resuspended to a concentration of 2 x 10^7 cells/ml in RPMI with 2% autologous plasma (DeKoning and Kaattari, 1991). Aliquots of 50 µl of cells plus 50 µl of 2% plasma in RPMI were cultured in individual wells of a 96 well flat bottom tissue culture plate (Corning Glass Works, Corning, NY). Ten µl of additional cells (2 x 10^5) were added in the suppression assays. Lipopolysaccharide (LPS), or TNP-LPS in appropriate concentrations were then added in 10 µl volumes. Cells were incubated in an incubator culture chamber (Model 624, C.B.S. Scientific Co., Del Mar, CA) at 17°C within a blood-gas environment containing 10% CO_2, 10% O_2, and 80% N_2.

Mitogen assay. Twenty hours prior to harvest, 10 µl (1 µCi) of ^{3}H-thymidine, specific activity of 6.7 Ci/mmole (ICN Biomedicals, Inc., Irvine, CA), diluted in RPMI was added to each well. Cells were harvested as previously described by Kaattari and Yui (1987). Results are expressed as cpms or stimulation indices (cpm of cultures with mitogen / cpm of cultures without mitogen).

Plaque forming cell assay. Antigen stimulated (TNP-LPS) cultures were harvested on day 9 of culture. The culture plates were centrifuged for 5

minutes at 500 x g. Culture supernatants were removed and cells were resuspended in 200 µl RPMI per well. Cells secreting anti-TNP antibody were then detected by methods previously described by Kaattari and Yui (1987). Results are expressed as plaque forming cells/ culture.

Irradiation studies. Cells from the peripheral blood, anterior kidney and spleen were harvested as described above. Single cell suspensions were prepared, washed and resuspended at 2 x 10^7/ml. Aliquots of cell suspensions were exposed to different doses of cobalt 60 gamma irradiation. Doses of 400, 800 and 2000 rads were used. Cells were then washed and resuspended to 2 x 10^7/ml. Cells that were not exposed to irradiation were similarly aliquoted, washed and resuspended at the same time. The effect of the irradiation on these cells was assessed in the LPS mitogen response.

Statistical analysis. Different statistical analyses of variance were used for the assays. The nonparametric Kruskal-Wallis test (a distribution-free, rank-sum procedure) was used to assess AKC effects on the PBL and spleen mitogen and PFC responses. Separate analyses were performed comparing AK to PB and SP in evaluating the effect of adding 20% of the respective cell population (Fig. 1 and Table 1).

A least squares linear regression approach was employed to examine the relationship between the AK stimulation indices and the degree of AKC suppression of the PBL mitogen response. Stimulation indices were defined as the ratio between the mean cpm of triplicate cultures with mitogen and the mean cpm of cultures without mitogen. A square root transformation was applied to the suppression value (% suppression / 100) to stabilize the variances prior to applying an analysis of variance to assess the significance of the relationship.

The statistical significance of the effects of irradiation on the PBL, SP or AK LPS mitogen response (Fig. 4, and Table 2) was examined using a logistic regression approach for categorical data. The response, as represented by stimulation indices, of each cell type (PBL, SP, AK) to the mitogen LPS was categorized as increased, decreased, no change, or no response. Chi-square analysis of variance was performed to determine significance.

RESULTS

Suppression of the PBL and splenic LPS mitogen responses by autologous anterior kidney cells (AKC). The inhibition of both peripheral blood (PBL) and spleen leukocyte proliferation upon addition of autologous AKCs was examined. Figure 4.1A depicts the suppression of the PBL response to the mitogen, LPS, when 2 x 10^5 AKC are added to 1 x 10^6 PBLs. When the same number of PBLs are added to the PBL cultures, no significant inhibition of mitogenesis is observed ($p<0.05$). Figure 4.1B also shows suppression of spleen cell cultures upon addition of AKC. When 2 x 10^5 PBLs are added to spleen cell cultures, no significant suppression is observed.

As a final control, anterior kidney cells were cultured with either 20% PBLs, or AKCs (Fig. 4.1C). Addition of PBLs to the AK cell cultures, did not result in significant suppression when compared to AKC addition to AK cell culture. Addition of AKCs to AK cultures resulted in the statistically significant suppression of the AK mitogen response. Thus, while PBLs had no significant effect on either PBL, spleen, or AK cell cultures, AKCs demonstrated significant suppression in each culture.

Correlation between the AK LPS mitogen response and the degree of AKC suppression of the PBL LPS mitogen response. Our studies have revealed that AK cell cultures often exhibit significantly lower levels of mitogenesis in response to LPS than do PBL, or splenocytes, from autologous fish (Fig. 4.2). The possibility was addressed that this substantially lower proliferative capacity may be the result of the same suppressive mechanisms observed when AKCs are cocultured with autologous PBL.

A value representing the degree of PBL suppression manifested by each AKC preparation was plotted against the stimulation index (SI) of the corresponding AK cells responding to LPS (Fig. 4.3). The values used were obtained as described in the materials and methods section. Regression analysis of this data revealed a statistically significant inverse relationship between the degree of suppression and the proliferative potential of the AK cells. This relationship predicts that with decreased proliferative activity in the AK, there is an increase in the ability of AKC to suppress the PBL mitogen response.

AKC effects on the PBL and splenic TNP-LPS plaque forming cell response. The effect of AKCs on the PBL and spleen plaque forming cell (PFC) response to the T-independent antigen, TNP-LPS, was also examined. As opposed to the proliferative asssays, AKCs appeared to only exhibit suppressive activity against splenocytes (Table 4.1). At 2×10^5, the AKC effect on splenic PFCs was comparable to the effect on mitogenesis, virtually uniform suppression. In contrast, there were few incidences of significant suppression (3/16) of the PBL PFC response, six cases of enhancement of the antibody response, and seven instances of no effect upon addition of AKC. However, overall it was determined there was no significant difference between these effects, and thus AKC generally had no effect on the PBL PFC response ($p<0.05$). The control, represented by addition of 2×10^5 PBL to either PBL, spleen, or AK cell cultures, did not result in any significant alteration of the AK cell response.

Effects of irradiation on the mitogen response of the PB, SP, and AK. Since certain suppressor cells have been shown to be sensitive to low dose irradiation (Anderson and Warner, 1976; Doria et al., 1982), the effect of cobalt 60 gamma irradiation exposure on the mitogenic response of

AK cells was examined. To determine if a radiosensitive suppressor cell population exists in either the AK, PB, or SP, an irradiation dose response was performed on cells from each organ. After irradiation, cells from each organ were examined for their mitogen response to LPS (Fig. 4.4). Results from the mitogen responses were recorded as an increase (I), decrease (D), or no change (NC) in proliferation, or no response (NR) after low irradiation doses of 400 - 800 rads (Table 4.2). Results demonstrated there was a significant difference between organ mitogen responses after irradiation ($p<0.05$). At doses of 400 - 800 rads, PBL and SP cells demonstrated an increase in proliferation in response to LPS, while no statistically significant enhancement of the proliferative response occurred in the AK. Thus, the AK seemed impervious to the effects of irradiation at these doses.

DISCUSSION

The results presented here demonstrate that the anterior kidney of the coho salmon houses a natural regulatory cell population with suppressor activity. The anterior kidney has been shown to be both functionally and morphologically heterogeneous in its constitution of cell types. Morphologically, monocytes, macrophages, lymphocytes, plasma cells, granulocytes, and erythrocytes have been identified in kidney cell suspensions (Yasatuke and Wales, 1983; Bayne, 1986; Zapata and Cooper,1990). Functionally, both mature and immature red and white blood cells have been observed (Smith et al., 1970; Zapata, 1979). Both histological and functional evidence support the hypothesis that the anterior kidney represents the primary hematopoietic organ in the teleost, and it has been compared to mammalian bone marrow in function (Zapata, 1979; Irwin and Kaattari, 1986; Zapata and Cooper, 1990). Being the primary hematopoietic organ, the cells of the anterior kidney would be expected to be under complex and strict immunoregulation, as has been observed in the mammalian counterpart to the anterior kidney; the bone marrow (Kincade et al., 1989).

As observed in the salmonid, anterior kidney cells (AKC), when cocultured with either peripheral blood leukocytes or spleen cells, suppressed the proliferative response to LPS, as shown in figure 4.1. This suggested that a natural effector cell population exists within the anterior kidney with a potential immunoregulatory role. Figure 4.2 shows the characteristic lower mitogen response of the anterior kidney as compared to the peripheral blood. Although we cannot dismiss the hypothesis that the lower response in the AK may simply be due to a lower number of

lymphocytes capable of responding to the mitogen, we propose rather, this lower responsiveness is due, at least in part, to internal regulation mediated by the suppressor cells in their native location.

More direct evidence to support this can be derived from the regression analysis that was performed to determine if there was a relationship between the percent AKC suppression of the PBL response and the AKC's ability to respond to mitogenic stimulation. The regression analysis of this data revealed a statistically significant inverse relationship between the degree of suppression and the proliferative potential of the AK cells. This relationship predicts that with an increase in AKC suppressor activity, there is a decrease in the AKC response to mitogen. Thus if we assume this decrease in AK proliferation is due to a suppressor activity, we can postulate that this signifies the active operation of a natural suppressor cell population within the anterior kidney which can downregulate the mitogen response not only of AKCs, but of peripheral blood and spleen lymphocytes as well.

When these same cultures were examined for their plaque forming cell response to TNP-LPS, peripheral blood lymphocytes and spleen cells responded differently. While AKCs significantly suppressed the spleen PFC response in the majority of experiments, the PBL antibody response was generally enhanced or unaffected upon addition of AK cells. The observation that antibody production is not suppressed in the PBL response while it is suppressed in the spleen response, adds another level of complexity to this phenomena. It is possible that there may be differences in the susceptibility of PBL versus splenic B cells to suppression by AKCs.

AKCs may have distinct effects on separate B cell subpopulations or different stages of B cell differentiation. Peripheral blood and spleen may

differ in the number of B cells within a given subpopulation or differentiation stage. The suppressor cell may inhibit the proliferative phase of T-independent B cell activation, similar to the inhibition of mitogen-induced proliferation. The difference between PBL and SP cell responses may then be explained if some PBLs did not need to proliferate before antibody production, whereas all splenic B cells required proliferation.

Alternatively, mitogen-induced proliferation (LPS) and T-independent antigen-induced proliferation (TNP-LPS) may not be one and the same process. TNP-LPS is thought to stimulate PFC production solely by focussing the mitogenic signal onto the B lymphocyte. However, if this were so, the PFC response to TNP-LPS should be reduced to a similar degree as the LPS mitogen response. This is not the case. Perhaps a dual signal, of TNP and LPS, is physiologically perceived as different than the one signal of LPS, and the former signal renders the cell somehow resistant to AKC suppression. This hypothesis, however, necessitates a corollary hypothesis that there at least two B cell subpopulations, one which perceives the dual signal and predominates in the blood, while the other relies solely on the mitogenic signal and predominates in the spleen.

Another model enlists the involvement of a second cell type, which is required for differentiation of the B cells. This cell may be present in both the peripheral blood and spleen, but may also differ in its sensitivity to AKC suppression. In the spleen this cell may be inhibited by AKC, resulting in inhibition of the PFC response, while in the blood, it may be resistant to AKC activity, allowing differentiation to continue even with diminished proliferation.

The similarities, and potential correlations, between the teleost anterior kidney and the mammalian bone marrow function may serve to

explain our observations. As stated above, both appear to be the primary sites of hematopoiesis, possess heterogeneous cell types and maturities, and demonstrate the potential for suppressor and cytotoxic activity. Antigen-nonspecific cytotoxic activity found predominantly in the anterior kidney has been observed in fourteen species of fresh water fish by Hinuma et al. (1980). Later Evans and coworkers (1984), proposed these cells were the evolutionary precursor to mammalian natural killer (NK) cells. Our data provides evidence of a suppressor cell population located in the anterior kidney, which may or may not be related to the cytotoxic cell described above.

In the mammalian system, natural killer cells (NK), natural cytotoxic cells (NC), and natural suppressor cells (NS) have all been shown to have separate and unique functions, while they all are recognized as members of the large granular lymphocyte (LGL) regulatory family (Maier et al., 1986; Maier et al., 1989). It is important to point out that although these cell types have been shown to possess unique functions, the relative scarcity of cell markers that would positively distinguish these cells makes it difficult to definitively assign a specific activity to one particular cell type. Thus, as in the teleost, these cell types have not been clearly differentiated. There are several cells within the mammalian LGL family that appear to have suppressive activity, such as NK, NS, and possibly NC cells. At this time, it is impossible to speculate whether the suppressive activity is mediated by a cytotoxic cell.

In comparing the observed AKC suppressor activity to mammalian suppressor cell types, there are strong similarities to the mammalian natural suppressor cells (NS). Both cells are most prominent in tissues undergoing considerable hematopoiesis, such as neonatal spleen and adult bone

marrow. Mammalian suppressors have been shown to block mitogen activation (Maier et al., 1985) and inhibit antibody responses (Layton et al., 1983). Additionally, they are able to suppress the mixed lymphocyte reaction (MLR) (Oseroff et al., 1984). NS cells are also characterized by their antigen-nonspecific, MHC-unrestricted suppression. NS cells do not appear to lyse their targets, but rather mediate suppression through the elaboration of a factor(s). The mammalian NS is relatively radioresistant, of a null phenotype and thought to be of the monocyte/macrophage lineage (Maier et al., 1986; Holda et al., 1990).

Table 4.2 shows low dose irradiation had no significant effect on the mitogen response of the AK, while PB and splenic cells gave an enhanced mitogen response after exposure to the same doses of irradiation. These data suggests the presence of a radiosensitive suppressor cell present in the PBL and SP cell populations, possibly reminiscent of the mammalian T suppressor which is known to be radiosensitive (Dorf and Benaceraff, 1984; Sherr and Dorf, 1982). However, this putative teleost T cell may differ from mammalian T suppressors, in that the latter are usually antigen specific and MHC-restricted. In our system, we do not use antigens to invoke this type of response, and thus would not expect to see evidence of T cell suppression.

In summary, we have presented data supporting the existence of a natural regulatory cell population in the anterior kidney with suppressor activity. We propose this to be the first demonstration in fish of a cell type analogous to the natural suppressor (NS) cell in mammals. Development of cell surface-specific markers in the salmonid will do much to characterize this cell. Although further characterization of this activity is required, this awaits better purification and identification methods which are still lacking even in the mammalian system, due to a lack of cell surface markers for

these cells which assume the null phenotype. Future studies might include the examination of function; whether the cell is lytic or not, whether there are soluble factors which mediate the suppression, as well as phenotypic characterization.

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Fig. 4.1 **Anterior kidney cell suppression of the mitogen response.** Cells were cultured in the presence of LPS at a concentration of 200 µg/ml. Cultures of either A) peripheral blood leukocytes, B) spleen cells, or C) anterior kidney cells consisting of 1 x 10^6 cells/ well were examined for their mitogen response either alone (■), or upon addition of 2 x 10^5 PBLs (▨) or AKCs (▩). Cultures were tested in triplicate, cells were incubated for 7 days at 17°C. Error bars represent one standard deviation. Cultures in the absence of mitogen did not exceed a mean of 2,508 ± 1,175 cpm.

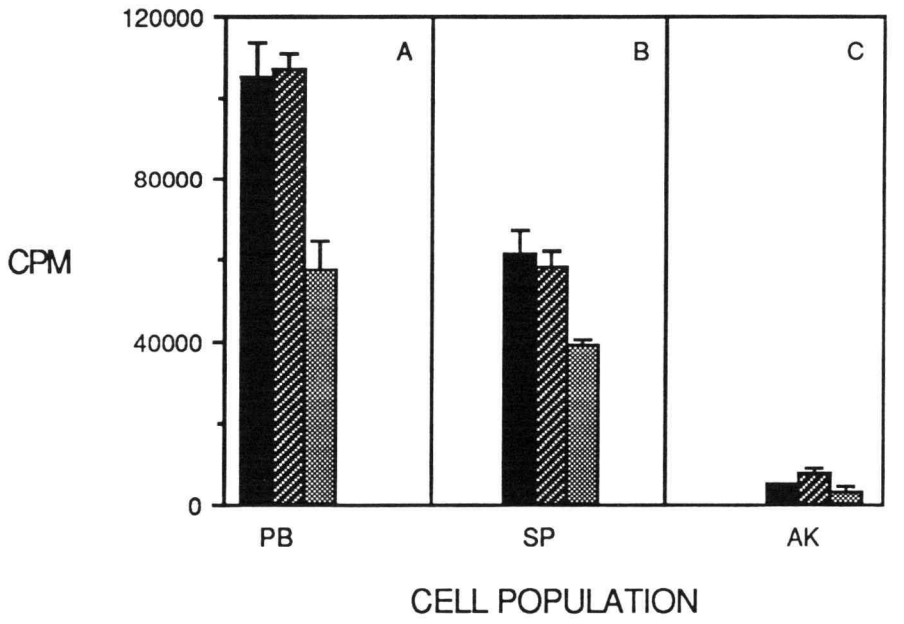

Fig. 4.2 **Comparison of PBL, AK, and SP responses to the mitogen, LPS.** The mitogen response of peripheral blood leukocytes (■), anterior kidney cells (▩), and spleen cells (□) when cultured in the presence of LPS, is shown. Results from three individual fish are demonstrated which is representative of >25 experiments. Cultures were tested in triplicate, cells were incubated for 7 days at 17°C. Error bars represent one standard deviation. Cultures in the absence of mitogen did not exceed a mean of 3,504 ± 1,123 cpm.

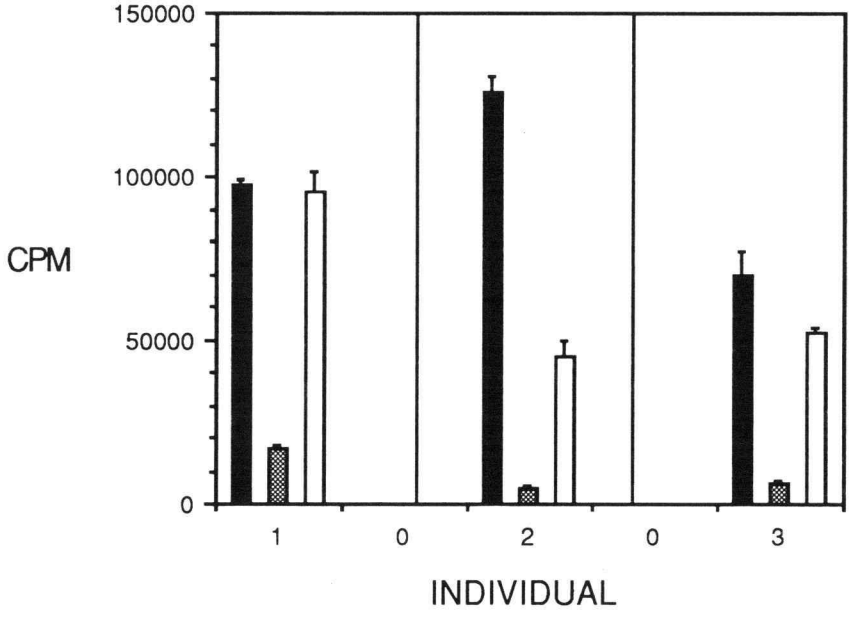

Fig. 4.3 **Relationship between observed AKC suppression of the PBL response and the AK mitogen response.** Data points represent 27 individual experiments. The observed suppression data upon coculture of AKC with PBLS is transformed as described in the materials and methods section and plotted against the respective AK stimulation indice (SI) value. Cultures were tested in triplicate, cells were incubated for 7 days at 17°C.

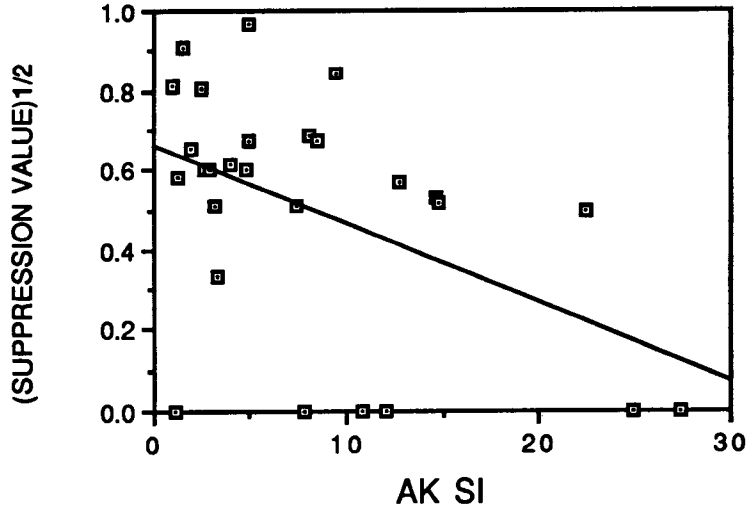

Fig. 4.4 **Irradiation effects upon the mitogen response of peripheral blood leukocytes, anterior kidney cells or spleen cells.** Single cell suspensions of peripheral blood leukocytes (A), spleen cells (B), and anterior kidney cells (C) were exposed to varying doses of gamma-irradiation, or no irradiation. Cells were subsequently plated with LPS, and their mitogen response determined. Results are expressed as percent of nonexposed control response. Cultures were tested in triplicate, cells were incubated for 7 days at 17°C. Cultures in the absence of mitogen did not exceed a mean of 1,792 ± 466 cpm.

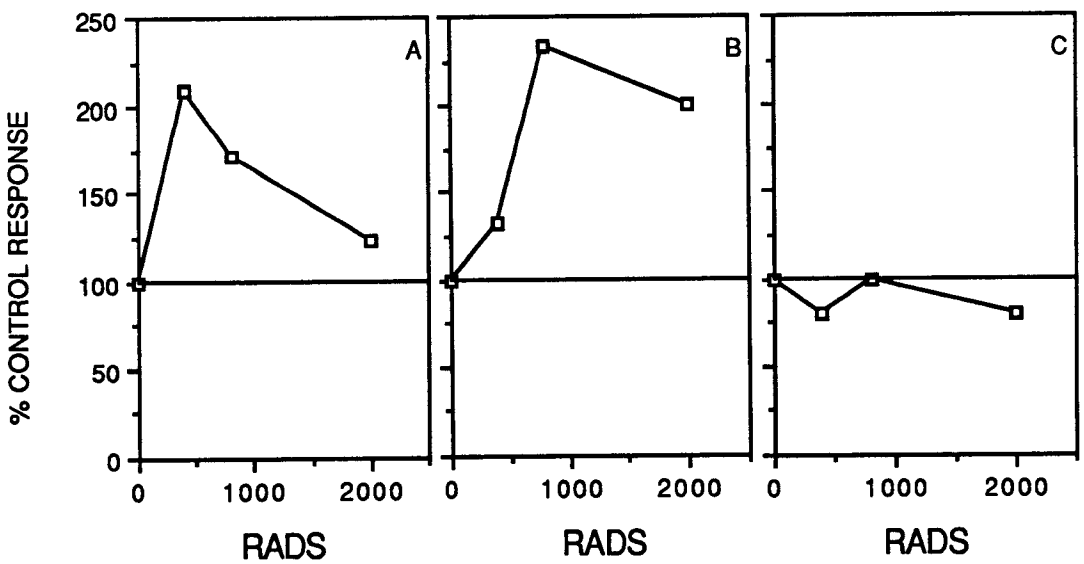

Table 4.1. **Effect of AKC on mitogen and antibody responses in PBL and spleen.** Suppression indicates >10% suppression of the control response. No effect indicates no significant difference from the control response. Enhanced indicates >10% enhancement of control response.

	Mitogen Response		Antibody Response	
Effect	PBLs	Spleen	PBLs	Spleen
Suppressed	18 / 24	17 / 17	3 / 16	12 / 16
No Effect	6 / 24	0 / 17	7 / 16	1 / 16
Enhanced	0 / 24	0 / 17	6 / 16	3 / 16

Table 4.2. **Irradiation effects on the LPS mitogen response of peripheral blood leukocyte, spleen cell, and anterior kidney cell cultures.** Results were obtained from 10 peripheral blood samples, 10 anterior kidney samples, and 8 spleen samples. I) Indicates significant Increase in the mitogen response after exposure of cells to 400-800 rads as compared to the nonexposed control response. D) Indicates significant decrease in the mitogen response after exposure of cells to 400-800 rads as compared to the nonexposed control response. NC) Indicates no change in the mitogen response after exposure of cells to 400-800 rads as compared to the nonexposed control response. NR) Indicates no significant response to the mitogen, when compared to unstimulated controls.

Response	PBL	SP	AK
I	9	6	3
D	1	0	3
NC	0	2	2
NR	0	0	2

CHAPTER 5

CONCLUSIONS

The commonality of the studies in this thesis is based upon the pursuit to improve and redefine the *in vitro* culture of salmonid leukocytes. One clue that lead to the supposition that we did not as yet have an optimum culture system, was the frequent occurence of 'nonresponders'. These nonresponders were defined as individuals whose leukocytes were unable to proliferate *in vitro* in response to a given mitogen. Previously it was assumed that nonresponders were simply an inherent part of the outbred population of fish we worked with. However, these studies demonstrated that wherein nonresponsiveness was observed, it was not due to an absence of responding cells, but rather due to suboptimal culture conditions.

In chapter 2 it is demonstrated that for rainbow trout peripheral blood leukocyte cultures, autologous or homologous rainbow trout plasma greatly improves mitogen responsiveness when compared to the traditionally used fetal bovine serum, or other mammalian serum sources. Repeatedly, when the same cells were compared for their mitogen responses after culture either in fetal bovine serum or rainbow trout plasma, plasma supplemented cultures demonstrated an enhanced mitogen response, in some cases up to 60 times the response observed in fetal bovine serum.

Another affirmation of the improved culture conditions was the altered kinetic response. Leukocytes cultured in plasma demonstrated a prolonged and enhanced kinetic response to the mitogen lipopolysaccharide. In trout,

this resulted in a peak response time of twice the duration of the peak response of leukocytes cultured in fetal bovine serum. This supported our contention that fetal bovine serum did not provide the necessary or essential components for trout leukocyte *in vitro* culture, and/or may contain components toxic or inhibitory to these cells.

We then sought to determine if this phenomena was limited to rainbow trout. In chapter 3, mitogen responses of leukocytes from coho and chinook salmon were examined when cultured in autologous plasma or fetal bovine serum. It was determined that again, plasma from either species resulted in an enhanced mitogen response when compared to fetal bovine serum. Interestingly, each species demonstrated a different optimal plasma concentration; for trout it ranged from 2 - 10%, for coho above 2% could prove toxic, and for chinook the optimal concentration appeared to be approximately 0.5%.

In keeping with the theme of improving and redefining the culture conditions for salmonid leukocytes, plasma was next examined to determine if it would also yield an enhanced antibody response, as well as an enhanced mitogen response. Again it was observed that fetal bovine serum resulted in suboptimal immune responses, while plasma did not only enhance proliferation of leukocytes, but differentiation and antibody production as well. This finding was extended to the anterior kidney and spleen cell responses in addition to the initial characterization performed with peripheral blood cells.

In a closer examination of plasma effects on leukocytes, it was observed that the enhanced mitogen response was specific. In the absence of mitogen the counts per minute (cpm) were consistently low, lower in fact than the response seen with fetal bovine supplemented cultures. We

demonstrated, as others (Etlinger et al., 1976a,b; Tillit et al., 1988), that fetal bovine serum can possess mitogenic activity in and of itself, which can stimulate leukocytes nonspecifically. Overall, we and others have demonstrated fetal bovine serum can have variable and deleterious results on leukocyte cultures, ranging from stimulation in the absence of mitogens to inhibition of both mitogen and antibody responses.

By substituting salmonid plasma for fetal bovine serum as the primary serum supplement for our salmonid leukocyte cultures, we felt we now had an optimal culture system. Though we had demonstrated how this method improved mitogen and antibody responses, we sought to further examine plasma effects on leukocyte responses in an as yet unexplored area. We began an investigation into autologous regulation of leukocyte responses mediated by a putative suppressor population within the anterior kidney. As both histological and functional evidence support the longstanding hypothesis that the anterior kidney represents the primary hematopoietic organ in the teleost, the anterior kidney would be suspected to be under complex and strict immunoregulation. The cells governing this immunoregulation, if any, are as yet unidentified, but by deduction would be naturally occurring, and antigen-nonspecific.

In chapter 4 it was demonstrated that addition of anterior kidney cells (AKC) to either autologous peripheral blood leukocyte or spleen cell cultures suppressed the mitogen response significantly. The AKC suppression of the peripheral blood response was found to be significantly correlated with the AKC's ability to respond to mitogenic stimulus. The correlation predicted that with an increase in AKC suppressor activity, a decrease in the AKC mitogen response was observed. Thus if we assume this decrease in the AKC mitogen response is due to a suppressor activity, we can postulate that

this signifies the active operation of a natural suppressor cell population within the anterior kidney which can downregulate the mitogen response not only of AKCs, but of peripheral blood and spleen lymphocytes as well.

When these same cultures were examined for their plaque forming cell response to TNP-LPS, peripheral blood leukocytes and spleen cells responded differently. While AKCs significantly suppressed the spleen PFC response in the majority of experiments, the PBL antibody response was generally enhanced or unaffected upon addition of AK cells, adding another level of complexity to this immunoregulatory phenomena. Several different models were proposed in the discussion section of chapter 4 which may account for this observed difference in antibody responses between the PBL and spleen. Although characterization of this suppressor population is obviously incomplete, and thus speculations on the mechanisms of this suppression are premature, it may be that AKCs have a differential effect on separate B cell subpopulations or separate B cell activation states. Peripheral blood and spleen may then differ in the number of B cells in a given subpopulation or activation state.

Also discussed in chapter 4 is the proposed identification or classification of these AKC suppressors. Once more, comparisons are made between the salmonid and mammalian immune system because of the extensive research in the mammalian system which has yielded clearer definitions of cell types. There are several components to the mammalian natural immune system, all of which have been proposed to play some part in the immunoregulation of the humoral response. However, upon a thorough review of the literature, when comparing the observed AKC suppressor activity to mammalian suppressor cell types, the strongest

correlations, in my opinion, suggest this activity is related to the mammalian natural suppressor (NS) cells.

Mammalian NS cells are characterized by their antigen-nonspecific, MHC-unrestricted suppression. These cells are most prominent in environments of considerable hematopoiesis, such as neonatal spleen and adult bone marrow. In this respect, it is compelling to point out that the anterior kidney of the teleost has long been considered to be the primary hematopoietic organ and the equivalent of the mammalian bone marrow. Other similarities between these cell types have been discussed, and in summary, it has been proposed that this may be the first demonstration in fish of a cell type analogous to the natural suppressor (NS) cell in mammals.

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APPENDIX

APPENDIX 1

REAGENTS AND BUFFERS

Benzocaine. Fish were anesthetized by immersion in a benzocaine bath of approximately 2 ml stock solution / 4 liters of water. The stock solution was prepared by dissolving 10 grams ethyl-p-aminobenzoate in 100 mls of 95% ethanol (Kaattari and Irwin, 1985).

Cacodylate buffer. A solution of 0.28 M Cacodylate buffer was prepared as described by Rittenberg and Amkraut (1966), by dissolving 3.82 g /100 ml cacodylic acid (Sigma, St. Louis, MO) in distilled water. 1N sodium hydroxide was used to adjust the pH to 7.0. Cacodylate buffer was stored at 4°C for no longer than one month.

Modified barbitol buffer (MBB). A stock solution of 5X MBB was prepared by dissolving 1 vial (0.05 moles sodium barbital, 0.01 moles barbital) barbital buffer (Sigma, St. Louis, MO) in one liter of distilled water at room temperature with constant stirring. Anhydrous calcium chloride (0.083g/l), magnesium chloride (0.508 g/l), and sodium chloride (42.5 g/l) were then added, and the pH adjusted to 7.4. The 5X stock solution was autoclaved and stored at 17°C. A 1X working solution was prepared by dilution with filter sterilized saline (8.7 g/l).

Phosphate buffered saline. Phosphate buffered saline (PBS) was prepared by dissolving monobasic potassium phosphate, KH_2PO_4 (1g/l) and

dibasic sodium phosphate-7 hydrate, Na_2HPO_4-$7H_2O$ (17.8 g/l) in one liter of distilled water. To this solution sodium chloride (8.5 g/l) was added and the pH adjusted to 7.4.

Trinitrophenylated lipopolysaccharide (TNP-LPS). TNP-LPS was prepared by the method of Jacobs and Morrison (1975). *E. coli* serotype 055.B5 lipopolysaccharide (161 mg) was suspended in cacodylate buffer (8.05 ml), with constant stirring. The pH of the solution was adjusted to 11.5 with 10 N sodium hydroxide. The tube was foil-wrapped and picrylsulfonic acid (96mg dissolved in 8ml cacodylate buffer) was added dropwise with constant stirring. The solution was stirred for two hours at room temperature. This solution of TNP-LPS was then dialyzed against 4 (1 l) changes of saline, and one (1 l) change of RPMI-1640. TNP-LPS was pasteurized for 45 minutes at 70°C. Stock TNP-LPS was stored at 4°C, and diluted in RPMI just prior to use.

Complement. Sera was obtained from spawned adult steelhead trout and used as the source of complement. Blood was collected in 50 ml centrifuge tubes, held on ice and allowed to clot overnight at 4°C. The sera was removed and pooled, then divided into aliquots which were stored at -70°C until used. Complement was diluted with MBB prior to use in the plaque forming cell assay.

Sheep red blood cell (SRBC) haptenation. Trinitrophenylated SRBCs were prepared using a modification of a method described by Rittenberg (1969). SRBCs are washed 3X in 1X MBB by centrifugation for 5 minutes at 2500 rpm. To the wet packed SRBCs (1 ml), a solution of 200 µl

picrylsulfonic acid in 3.3 ml cacodylate buffer is added dropwise. The tube is foil-wrapped and placed on a rotator for 20 minutes at room temperature. The SRBC solution is centrifuged, the supernatant removed and a solution of 0.0037 g of glycyl-glycine dissolved in MBB is added to block the remaining active sites. The cells are centrifuged again, supernatant removed, then washed 3X in MBB. The SRBCs are then resuspended to a concentration of 20% in MBB for final dilution.